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PHYSIOLOGICAL ZOÖLOGY

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INDUCED STERILITY[1]

(Eighteen figures)

M. F. GUYER AND PEARL E. CLAUS
Department of Zoölogy, University of Wisconsin

THE senior writer's (1922) earlier experiments with spermatotoxic sera were confined to males. In that study he found among other things that not only would spermatotoxic sera act *in vitro* but also *in vivo*, and that, furthermore, the blood serum of a rabbit injected intravenously with its own spermatozoa becomes toxic for the spermatozoa of all rabbits, including its own. The spermatozoa of such a rabbit are much less viable, both in normal serum and in spermatotoxic serum, than are normal spermatozoa. Presumably they have been injured *in vivo* by the spermatotoxic serum of the animal body to which they belong. In 1923 McCartney showed that female rats may be sterilized for a period of 2–22 weeks by the injection of spermatozoa or testicular extracts and that the injection of other organ extracts does not cause sterility. He believed the sterility to be due to spermatotoxins in the female genital tract, since, according to him, the oestrous cycle of the female was not affected by immunization with spermatozoa. McCartney also found that upon becoming fertile again after having been temporarily sterilized by subcutaneous injections of rat spermatozoa, the treated rats cast smaller litters than did normal rats.

[1] This study was begun under a grant from the Committee on Maternal Health of New York City, continued under one from the C. F. Burgess Research Laboratory, and finished under a University of Wisconsin Research Grant.

Pommerenke, a research assistant of the senior writer, in the meantime had undertaken a rather extensive series of experiments on the effects of sperm injections into female rabbits. Although his experiments were concluded several years ago they were not published until 1928. Pommerenke (1928) found, among other results, that not only the serum but the vaginal secretions of females injected with rabbit semen become toxic for rabbit spermatozoa; that infertility for a period of 6–25 weeks may follow the repeated injection of spermatozoa or of testicular extracts into female rabbits, although the oestrous cycle is not affected; and that even repeated intravaginal injections of rabbit spermatozoa into female rabbits may produce antigenic effects in both blood and vaginal secretions of such rabbits, although no conclusive evidence that sterility followed such intravaginal injections was obtained.

Various experiments in which endocrinal materials were employed are also of some significance in connection with problems of sterility. Thus, Gudernatsch (1915) discovered that thyroid substance administered to either male or female rats tended to prevent pregnancy, or if pregnancy occurred, to produce abortion; and Weichert (1930) found that, in rats, thyroid feeding caused resorption of young in some, abortion in others, and prolonged gestation with eventual delivery of dead young in still others. In rabbits fed thyroid substance, conception occurs, according to the investigations of Kunde, Carlson, and Proud (1929), but during the latter two-thirds of the gestation period, the embryos are absorbed.

Since the beginning of the present experiments in 1927 the authors have been endeavoring mainly to investigate further the possibilities of inducing sterility in female rats and rabbits, although certain experiments on males have made up part of the program. Inasmuch as the results of injecting spermatozoa and the cruder forms of testicular extracts are already well known through the observations of numerous investigators, attention was turned more particularly to the employment of testicular nucleoproteins, which from earlier experiments there was reason to suspect might act as inhibitors of fertility, and to extracts of the anterior lobe of the pituitary body. A commercial preparation of anterior lobe of the pituitary was used, but the nucleoproteins were specially prepared by the research chemist of

the department, Dr. Samuel Lepkovsky, and later, by Dr. H. L. Fevold. No attempt is made to review the literature on spermatotoxins as this has been done rather extensively in the papers of McCartney (1922) and Pommerenke (1928).

Preparation of nucleoprotein.—The nucleoproteins employed were concentrated from over 250 pounds of bull testicles. After removal of the tunics the testicles were ground and 0.7 per cent NaCl solution containing 0.1 per cent Na_2CO_3 was added, in the proportion of one volume of testical material to two volumes of the saline solution. After thorough shaking and standing over night in the icebox, the mass was centrifuged. The supernatant liquid was collected and precipitated with acetic acid. The nucleoproteins were then thrown down with the centrifuge and washed. They were next redissolved in dilute alkali (0.5 per cent Na_2CO_3), again centrifuged, and again reprecipitated and washed. This crude nucleoprotein was further refined by again dissolving and reprecipitating it twice.

Histological technique.—In general, Bouin's picroformol fluid was used in fixing ovaries and pituitary bodies. Ovarian sections were commonly stained with Delafield's hematoxylin; sections of the pituitary, with a modified Mallory's triple stain in which the acid fuchsin was a 10 per cent solution in anilin water. For study of the Golgi body, Ludford's modification of the Mann-Kopsch technique was found to be the most satisfactory.

EXPERIMENTS

In all the following experiments the females were young rats or rabbits, each one of which had borne one litter of young and were therefore known to be fertile. Likewise, only males of tested fertility were used. While the primary object was to determine the effect of testicular nucleoproteins, of spermatotoxins, and of anterior lobe of the pituitary on fertility, extracts of (1) bull epididymis, (2) crude bull testes, (3) salivary gland of the rabbit, and (4) mammary substance of the cow were also used, partly as controls and partly to discover if any of these substances influenced fertility when injected as solutions or suspensions. The injection experiments were all done with rats. In general, in the earlier experiments, three to five (some times more) intraperitoneal injections of 1 cc. each of the substance

to be tested were given at intervals of 3 or 4 days, and within a few days following the last injection the male was placed with the injected females. The rats were kept in cages, with 5–7 females and 1 male in each cage; each female rabbit was kept with a separate male.

1. *Testis and epididymis extract of rat.*—The combined epididymis and testis material from several rats were ground thoroughly, dried, and powdered. One cc. of an emulsion of 0.5 cc. of this powder in 10 cc. of normal saline solution was injected into each of 25 females on three separate occasions at intervals of 4 days. Males were placed with the females 3 days after the last injection. Within 6 weeks of the introduction of the males 21 of the 25 females had borne young or were pregnant, hence it is evident that the injected material was ineffective in producing any appreciable sterility. Inspection of vaginal smears from time to time showed no effect on the oestrous cycle.

2. *Mammary substance.*—Fifteen female rats were each injected intraperitoneally with 1 cc. of a suspension of 0.5 cc. of powdered mammary substance of the cow in 10 cc. of normal saline solution. Four injections were given at intervals of 4 days. Males were then introduced. At the end of 5 weeks 4 females had young; within the next 2 weeks 5 more bore young and 1 was obviously pregnant. Two of the females died and the remaining 3 were still without young 16 weeks after being placed with the males. The oestrous cycle seemed unaffected. See also control under 4.

3. *Infra-red rays.—Rats:* Ten female and 4 male rats were given daily exposures of $\frac{1}{2}$ hour each to infra-red rays at a distance which in $\frac{1}{2}$ hour raised the rectal temperature of a rabbit 2 or 3°. Fertility and oestrus seemed unaffected during the duration of the experiment which was continued for 32 days, until 8 of the 10 females had become pregnant.

Rabbits: With male rabbits, however, the result was different. During the winter and spring of 1927, 21 male rabbits of known fertility were rayed with infra-red. Exposures to the rays were for 30 minutes each time, repeated for each rabbit on 10 successive days. During the raying each rabbit was held in place in a lidless box with a curved false-bottom of coarse screening, separated about 3 cm. from the similarly curved, aluminum-lined true bottom. The latter

thus acted as a reflector. The light was placed 32 cm. above the rabbit. Rectal temperatures taken before and after each treatment showed that such half-hour exposures raised the temperature of the rabbits some 2–3°, and occasionally as much as 4°. An observed copulation of each male occurred within 6 days after the last raying, and

TABLE I

INFRA-RED RAY TREATMENT OF MALE RABBITS

Rabbit	Degrees of Temperature Rise in Ten Treatments		Days of Sterility to First Fertile Mating	No. of Young Produced	Remarks
	Lowest	Highest			
1.....	1.2	3	30	2	
2.....	1	2.8	.....	.....	Died after 7th treatment
3.....	2	3	63	5	
4.....	1.5	3	81	4	One testis removed after 20 days
5.....	2.3	4	92	3	
6.....	2	3.2	87	4	
7.....	2	3	22	3	Fertile 22 days, after treatment; later permanently sterile
8.....	1.8	3.8	89	3	One testis removed after 20 days
9.....	1.8	3.4	76	6	
10.....	2	3.8	114	7	
11.....	2	4	.....	.....	Permanently sterile
12.....	1.7	3.6	.....	.....	Died a week after final treatment
13.....	2	3.2	77	4	One testis removed after 20 days
14.....	2	3	97	6	
15.....	2	2.8	86	4	
16.....	3	3.8	.....	.....	Permanently sterile
17.....	2	3.2	81	2	One testis removed after 20 days
18.....	2	3.4	78	6	
19.....	2	4	.....	.....	Permanently sterile
20.....	1.8	3.6	92	3	
21.....	2	3.2	76	1	
Average	.....	.....	77.5	.....	.....

the animals were mated at weekly or more frequent intervals. In the cases of rabbits Nos. 4, 8, 13, and 17 (Table I), one testis was removed from each, 20 days after the last treatment, and prepared by the usual paraffin section method for microscopical examination. Such inspection showed the testes to be somewhat flabby and shrunken with much degeneration of spermatocytes and spermatids but with spermatogonia apparently unaffected. Occasional traces of

spermatozoa were in evidence in Nos. 8 and 17, but spermatozoa seemed to have disappeared entirely from Nos. 4 and 13. Samples of semen recovered from matings of these rabbits just before their unilateral castration were devoid of spermatozoa. All four of these matings also proved sterile. Nos. 11, 16, and 19 apparently became permanently sterile, for although kept under observation for over a year with abundant opportunity to mate they never produced young.

The individual results of the treatment of these 21 male rabbits by infra-red rays are summarized in Table I. Leaving out of consideration the animals permanently sterilized (Nos. 11, 16, and 19) and the 2 which died, the average time of sterility, reckoned from the date of the last ray treatment until the first mating that yielded young, was about 77.5 days, with the extremes at 30 and 114 days, respectively.

There is no reason to believe that the effect of such infra-red treatment is other than one of temperature. At every treatment the internal temperature of the animal was raised some 1–3° and this in itself, as Moore (1924) and others have repeatedly shown, is sufficient to produce sterility in male mammals. In a recent paper Cunningham and Osborn (1929), working with rats, have shown the parallel sterilizing effects of infra-red rays and of heated air when similarly applied directly to the testes. As a matter of fact, spermatogenesis in rabbits seems easily upset by any one of varied experiences. In connection with other experiments carried on by the writer the lenses of the eyes in a number of male rabbits were needled. It was planned to mate them about 15 days after the operation but with rare exceptions fertile matings could not be secured from them under 2 or 3 months. Again male rabbits which have been injected with typhoid vaccine or with living typhoid bacilli seem generally to pass through a period of infertility.

4. *Dried epididymis.*—The epididymis was removed from the testes of bulls with as little connective tissue as possible. Most of the ducts were filled with spermatozoa. In fact, the reason for using epididymus was this richness in spermatozoa. This seemed to be one of the best ways to secure a concentrated solution of spermatozoa to use as antigen. The material was ground thoroughly three or four

times then diluted into a thin syrupy mass with normal saline solution to which had been added a trace of Na_2CO_3. After standing for 24 hours the fluid part was poured onto glass plates so that the water might evaporate. The dry residue was scraped off and powdered. One gram of the powder was thoroughly mixed with 20 cc. of normal saline solution, and 1 cc. of this solution was used for each injection (see Table II).

TABLE II

FEMALE RATS INJECTED WITH EXTRACT OF BULL EPIDIDYMIS

Twenty rats; dried epididymis extract. Seven injections given Nov. 26, 1926—Jan. 4, 1927. Males introduced Jan. 10, 1927.

Rat No.	Number and Date of First Young
1	4, May 2
2	Died
3	3, Apr. 4
4	5, May 8
5	Died
6	3, Apr. 10
7	Still sterile July 16
8	5, June 3
9	Still sterile July 16
10	5, Apr. 13
11	Died
12	Still sterile July 16
13	Still sterile July 16
14	4, Apr. 13
15	Died
16	5, June 7
17	Still sterile July 16
18	7, May 17
19	Still sterile July 16
20	4, July 3

Ten control rats; mammary gland extract. Seven injections given Nov. 26, 1926—Jan. 4, 1927. Males introduced Jan. 10, 1927.

Rat No.	Number and Date of First Young
1	6, Feb. 16
2	6, Feb. 8
3	5, Feb. 17
4	8, March 3
5	5, March 10
6	Died
7	None born by July 16
8	6, Feb. 27
9	7, March 2
10	7, Feb. 17

Twenty female rats were injected intraperitoneally with the mixture at about 5-day intervals between November 26, 1926, and January 4, 1927, each being given seven injections. A tested, vigorous male was added to each cage of 5 females on January 10, 1927. No young were born until April 4. Four litters were born in April; 3 in May; 2 in June; and 1 in July. Four of the females died without young. The remaining 6 were still sterile at the conclusion of the experiment July 16. The extract of dried epididymis apparently re-

tarded fertility decidedly in the majority of the females and rendered 6 sterile for the time the experiment was in progress.

Ten controls were injected with extract of bovine mammary gland. An inspection of Table II shows that the mammary substance interfered with fertility little if at all. One rat died and 1 remained sterile although in view of the fertility of the other 8 it is improbable that the injected mammary substance was the specific cause of the infertility. It is also observable that the number of young per litter is reduced in the animals treated with epididymis extract.

5. *Spermatozoa of the bull.*—The spermatozoa were secured by mincing epididymis and washing out the spermatozoa in normal saline solution, discarding all solid particles and evaporating the remaining milky fluid from plates of glass. The dried residue was powdered and 0.5 gram of it added to 20 cc. of salt solution was used for an injection mixture (Table III). Eighteen female rats were injected intraperitoneally with this mass at about 5-day intervals between November 26, 1926, and January 4, 1927. Six injections were given in all. A male was added to each cage of 6 females on January 10, 1927. No young were born until April 18. Three litters were born in April; 2 in May; 3 in June; and 2 in July. Two females died without young. The remaining 6 females were sterile at the conclusion of the experiment July 20. Inspection of Table III shows that the extract of dried spermatozoa delayed and diminished fertility, since

TABLE III

FEMALE RATS INJECTED WITH SPERMATOZOA OF THE BULL

Eighteen rats; six injections from Nov. 26, 1926, to Jan. 4, 1927. Males introduced, Jan. 10, 1927. Controls same as in Table II.

Rat No.	Number and Date of First Young	Rat No.	Number and Date of First Young
1	5, May 7	10	Died
2	5, Apr. 20	11	6, July 7
3	Still sterile, July 20	12	Still sterile, July 20
4	Died	13	Still sterile, July 20
5	3, Apr. 18	14	4, June 5
6	4, June 3	15	3, June 20
7	Still sterile, July 20	16	Still sterile, July 20
8	Still sterile, July 20	17	5, July 10
9	3, Apr. 22	18	4, May 10

only one litter numbered as high as 6. It apparently rendered sterile 6 of the 16 females which survived the experiment.

6. *Supernatant fluid of epididymis plus testicular feeding.*—Forty-one female rats were used in all. The epididymis was cut away from the testis leaving as little connective tissue as possible. The material was taken from bulls. In many cases the epididymis was filled with spermatozoa. The tissue was ground thoroughly and finally made into a soupy mass by the addition of normal saline solution to which a trace of Na_2CO_3 had been added. The mass was shaken from time to time during 24 hours and then allowed to settle. One cc. of the supernatant fluid was used for each injection.

The experiment was begun April 19, 1927, and continued till September 21. A few days after each rat had been injected for the third time, with intervals of 4 days between injections, 1 male was added to each cage of 5 or 6 females (Table IV).

After the first 6 weeks, the rats were fed on dried testicular material two or three times a week to determine whether or not sterility could be maintained through dosage by mouth in case the original treatment had induced it. The males were introduced about May 10, some a day earlier, some a day or two later. The first litter was born June 11. Another was born June 15. Seven litters in all were born in June; 5 litters in July; 6 in August; and 5 in September. Nine females died during the course of the experiment. The remaining 9 females remained sterile during the 5 months of the experiment and when killed at its conclusion they were found to have no young in their uteri. Of the 32 females which survived, all but 9 bore young at some period during the 5 months. In every case but one, however, the litters were small, numbering from 3 to 6. In one case there were 8. It would seem that the treatment with such supernatant epididymis material reduced or retarded fertility without completely stopping it in most of the animals, but prevented it in 9. The thought back of feeding dried testicular material was that perhaps a sterility once induced by means of spermatotoxins might be prolonged by oral administration of material containing the antigen for such spermatotoxins. No appreciable prolongation of the sterile period, however, seemed to have been effected by such feeding.

The 25 rats used as controls were injected each time with 1 cc. of

TABLE IV

INJECTION OF EPIDIDYMAL FLUID FOLLOWED BY FEEDING OF TESTICULAR MATERIAL

Forty-one female rats injected with epididymal fluid; fed testicular material. Three injections given from Apr. 19, 1927, to May 1, 1927; feeding began June 3.

Twenty-five control rats injected with rabbit salivary gland extract. Three injections given from Apr. 19 to May 1, 1927; feeding as in the other group.

	Male Introduced	Date and Number of First Young		Male introduced	Date and Number of First Young
Cage 1, 5 rats..	May 9	1. July 1, 3 2. Aug. 12, 4 3. Aug. 14, 4 4. Died 5. Sterile Sept. 21	Cage 1*a*, 5 rats..	May 9	1. June 20, 6 2. June 14, 4 3. June 16, 4 4. Died 5. Died
Cage 2, 5 rats..	May 9	1. July 3, 4 2. Sept. 2, 5 3. Died 4. Sterile Sept. 21 5. Aug. 17, 5	Cage 2*a*, 5 rats..	May 9	1. Died 2. June 29, 5 3. June 11, 5 4. June 21, 5 5. June 16, 4
Cage 3, 5 rats..	May 10	1. June 11, 3 2. June 17, 4 3. Sterile Sept. 21 4. Sterile Sept. 21 5. Died	Cage 3*a*, 5 rats..	May 9	1. July 5, 6 2. June 13, 6 3. Sterile Sept. 21 4. June 13, 5 5. July 5, 3
Cage 4, 5 rats..	May 10	1. July 4, 4 2. July 20, 5 3. Died 4. Died 5. Sterile Sept. 21	Cage 4*a*, 5 rats..	May 9	1. June 28, 5 2. Died 3. June 25, 5 4. July 6, 5 5. July 4, 4
Cage 5, 5 rats..	May 10	1. June 15, 4 2. June 17, 4 3. Sept. 11, 4 4. Sterile Sept. 21 5. June 20, 5	Cage 5*a*, 5 rats..	May 9	1. July 3, 7 2. June 20, 6 3. Sterile Sept. 21 4. June 17, 5 5. Sterile Sept. 21
Cage 6, 5 rats..	May 10	1. Aug. 3, 8 2. June 24, 3 3. Sept. 7, 3 4. Sept. 6, 6 5. Sterile Sept. 21			
Cage 7, 5 rats..	May 10	1. July 4, 3 2. Aug. 2, 5 3. Died 4. Died 5. Sterile Sept. 21			
Cage 8, 6 rats..	May 11	1. June 20, 3 2. Aug. 6, 4 3. Sept. 6, 4 4. Died 5. Sterile Sept. 21 6. Died			

an extract of dried rabbit salivary gland made up in the proportion of 1 gram of the dried gland to 20 cc. of normal salt solution. An inspection of Table IV shows that fertility was interfered with little if at all. While 3 of the 16 animals which survived the experiment remained sterile, the records of the others indicates that the sterility was probably not attributable to any antigenic effect of the salivary extract.

7. *Anterior lobe of the pituitary body.*—The substance of the anterior lobe of the pituitary was used partly in an exploratory way but also because the announcement of Evans and Long in 1921 concerning its interference with ovulation made it desirable to compare its effectiveness with that of spermatotoxins in the production of sterility. The desiccated anterior pituitary body prepared by Armour and Company, 1 gram of which represents 5 grams of fresh substance, was used in all experiments. The experiments were first started with 20 rats on November 6, 1926, but it soon became evident that the dosage of 1 cc. of a 1 to 10 dilution (1 gram in 10 cc. of normal saline with a trace of Na_2CO_3) was entirely too strong. A number of the rats died and the others were so incapacitated that they were abandoned. Tests on 5 additional rats showed that even a solution of 1 cc. of a 1 to 20 was not tolerated well and 0.75 cc. of a dilution of 1 part of dried material to 25 parts of fluid was finally settled upon, for the experiment. A new series of 20 rats was selected and the experiment was begun November 26 to run in parallel with those recorded in Table II. Injections were given every third day at first, but, later, every second day. A male of known fertility was added to each cage of 5 females after six injections had been given. The outcome is shown in Table V. The last injection was made on January 29, 1927. Four of the females (Nos. 1, 2, 8, 16) had died in the meantime. Of the remaining 16, only 1 (No. 6) bore young during the course of the treatment. The fact that there were only 3 young indicates that her fertility had been reduced. Three (Nos. 4, 9, 20) seemed to have been rendered permanently sterile for although constantly with males they had produced no young before June 1, 1927, when the experiment was discontinued. Three females (Nos. 5, 10, 13) were killed on January 25, 1927, in order to make microscopic examinations of their ovaries. More corpora lutea were present than one would find under normal conditions and in many cases

they were unusually large. In some the unshed ovum was discernible. The general picture in sections was essentially that described and figured by Evans in his 1924 Harvey Lecture and his interpretation

TABLE V

FEMALE RATS INJECTED WITH EXTRACT OF DRIED ANTERIOR PITUITARY BODY

Twenty Rats	Injections 1926–27	Male Introduced	Young Born	Remarks
1.....	Nov. 26, 29; Dec. 3, 6, 9, 12, 15, 20, 23, 26; Jan. 1, 3	Dec. 12	.....	Died, Jan. 6, 1927
2.....	Nov. 26, 29; Dec. 3, 6, 9, 12, 15, 20, 23, 26; Jan. 3, 7, 9, 11, 13, 15	Dec. 12	.....	Died Jan. 17, 1927
3.....	Nov. 26, 29; Dec. 3, 6, 9, 12, 15, 20, 23, 26; Jan. 17, 19, 21, 23, 25, 27, 29	Dec. 12	4, Mar. 5	.....
4.....	Same as No. 3	Dec. 12	.....	Still sterile June 1, 1927
5.....	Same as No. 3 to Jan. 23	Dec. 12	.....	Killed Jan. 25 for study of ovary. Numerous large corpora lutea, unshed ova
6.....	Same as No. 2 to Jan. 13	Dec. 15	3, Jan. 18	.....
7.....	Same as No. 3	Dec. 15	3, Mar. 20	.....
8.....	Nov. 26, 29; Dec. 3, 6, 9	Dec. 15	.....	Died Dec. 12, 1926
9.....	Same as No. 3	Dec. 15	.....	Still sterile June 1, 1927
10.....	Same as No. 3 to Jan. 23	Dec. 15	.....	Killed Jan. 25 for study of ovary. Numerous large corpora lutea, unshed ova
11.....	Same as No. 3	Dec. 16	3, Mar. 18	.....
12.....	Same as No. 3	Dec. 16	5, Mar. 18	.....
13.....	Same as No. 3 to Jan. 23	Dec. 16	.....	Killed Jan. 25 for study of ovary. Numerous large corpora lutea, unshed ova
14.....	Same as No. 3	Dec. 16	5, Mar. 22	.....
15.....	Same as No. 3	Dec. 16	5, Apr. 7	.....
16.....	Same as No. 3 to Jan. 27	Dec. 16	.....	Died Jan. 29, 1927
17.....	Same as No. 3	Dec. 16	2, Feb. 28	.....
18.....	Same as No. 3	Dec. 16	4, Mar. 17	.....
19.....	Same as No. 3	Dec. 16	3, Mar. 16	.....
20.....	Same as No. 3	Dec. 16	.....	Still sterile June 1, 1927

that luteal substance develops precociously and imprisons the ovum before it can be shed is possibly the correct one. He believes that both germ-cell injury and stimulation to luteal cell growth occur.

In our preliminary experimentation with the dosage of anterior

pituitary substance to use, however, 5 female rats which were set aside in one cage and given much smaller doses (0.25 cc. of a 1 to 20 dilution) gave no evidence of inhibition of ovulation. The indications were just contrary to this. The rats were injected respectively at 3-day intervals from November 6 to December 26. A male of known fertility was introduced into the cage December 3. Within less than 5 weeks each of the 5 females had borne young, the litters numbering 6, 7, 7, 9, 8, respectively. As matters stood at the termination of this first series of experiments with anterior pituitary substance, therefore, one could infer that light dosages favored, or certainly did not inhibit, ovulation, while heavier dosages prevented it. The possible explanation of this apparent contradiction came later in the discovery by the junior author (Claus, 1931) that a crystalline substance, or a substance associated with certain characteristic crystals, which induces precocious sexual maturity, can be extracted from the anterior lobe of the pituitary body, leaving a separate fraction in the residual substance which still inhibits ovulation; it induces excessive development of luteal substance. Apparently the anterior lobe contains two substances, one an ovulation stimulant, the other an ovulation inhibitor, and in dilute dosages of anterior lobe substance the former prevails. This may be the explanation of enhanced fertility reported after feeding anterior substance to birds (Clark, 1915) or following transplants of fresh anterior lobe substance in mice (Smith and Engle, 1927).

Our knowledge of the whole question of anterior lobe secretions, however, is still in the making. Fevold, Hisaw, and Leonard (1931), for instance, by means of aqueous pyridine extraction, have obtained two substances from the anterior pituitary: one a gonad-stimulating hormone which incites follicular growth, the other a luteinizing material. These two substances can hardly be the same ones isolated by the acidified alcohol method (Claus, 1931) since they are insoluble in acid alcohol or 99 per cent alcohol. Inasmuch as the gonad-stimulating substance of Claus was found to be obtainable not only from the pituitary anterior lobe but from various other sources as well, both plant and animal, it may be that, as suggested by her, it is a substance of more general nature than a specific anterior lobe hormone.

ANTERIOR LOBE EXTRACT AND THE OESTROUS CYCLE

In the experiments on anterior lobe extract set forth in Table V, the effect of the treatment on the oestrous cycle was not taken into account. It seemed desirable therefore to run a new series with the purpose in part of checking this aspect of the reaction and also for a more careful study of the manner in which the sterility was induced as compared with that resulting from the injection of testicular nucleoproteins as described later.

On February 9, 1928, 20 healthy fertile young female rats were selected for the experiment. Each was injected with 0.5 cc. of a suspension of 1 gram of desiccated anterior pituitary substance in 10 cc. of normal saline solution made slightly alkaline (8.5 grams NaCl plus 1.0 gram Na_2CO_3 in 1,000 cc. of distilled water). This was found to be too irritating. Sores appeared in the skin around the points of injection and the animals were left until March 3, 1928, to recover. Beginning then, vaginal smears of each of the 19 remaining rats were made on successive days. The results are shown in the form of graphs in Figure 1. The data for such graphs were, of course, first accumulated in tabular form. The four conventionally recognized stages in the usual 4- to 5-day oestrous cycle were recorded in our tables by letters thus:

Stage 1, nucleated cells, by the letter *N;*
Stage 2, scales (full oestrum), by the letter *S;*
Stage 3, disintegrating scales—some whites—by the letters *SW;*
Stage 4, white blood cells, by the letter *W*.

Hyphenated letters were used to indicate transitions between the several conditions.

On the fourth day, March 9, 1928, injections were begun. One gram of desiccated anterior lobe substance was shaken well many times during several hours with 20 cc. of a slightly alkaline normal saline solution. This was then centrifuged and, unless otherwise specified in the graphs, 0.5 cc. of the liquid portion was injected into each rat intraperitoneally. At first the extract was that of 1 gram of beef desiccated anterior lobe in 20 cc. of normal saline solution, later a 1 to 40 cc. solution was used. In the case of rats Nos. 16, 17, 18, and 19, after June 20, 1928, a crude acid-alcohol solution was used.

With occasional exceptions daily vaginal smears were made from

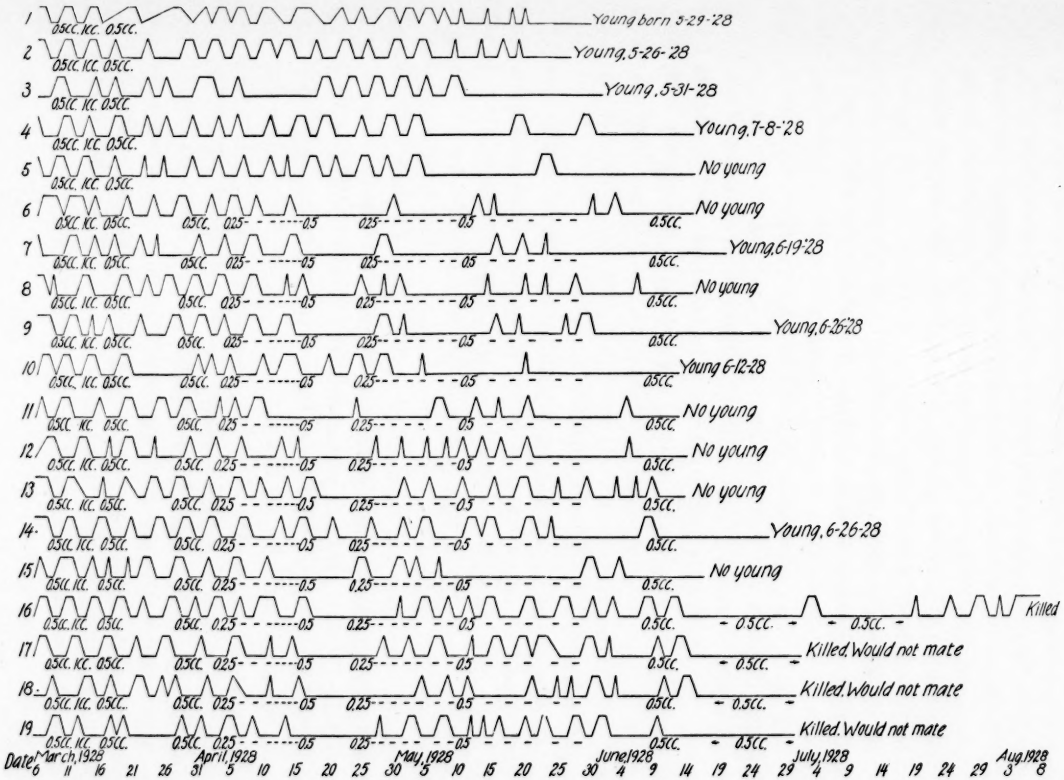

FIG. 1.—Graphs showing history of the oestrous cycles of 19 rats injected with anterior lobe extract. The upper level indicates oestrum, the lower level, dioestrum; the lateral spread signifies time in days. The dosage employed during a given period is indicated beneath each graph. Dates in 5-day intervals are shown at the bottom of the figure.

each rat and inspected under the microscope. The findings are recorded in Figure 1. The upper level of each graph indicates oestrum, shown by the presence of scales in the vaginal smears; the lower level, dioestrum, indicated by the presence of characteristic leucocytes. The lateral spread signifies time in days. The dosages varied as indicated in the graphs.

An inspection of these graphs shows that shortly after the injections were started, March 9, 1928, the females became irregular in their oestrous cycles with, in most, prolonged intervals of dioestrum, during more than $3\frac{1}{2}$ months of daily inspection. After 20 days of injection (March 29) 5 rats (individuals 1–5) in cage 1 were given no further injections. Within 5 days all had shown evidence of oestrum. The ensuing periods, however, as revealed from an inspection of the first five graphs of Figure 1, showed much irregularity with a tendency for the interoestrous period to be much longer than normal. In the meantime injections were continued with the other animals (individuals 6–19) until June 11, 1928.

On May 1, 1928, 52 days after injections were begun, males were introduced into cages 1 and 2, which contained individuals 1–10. In cage 1 were the 5 individuals (1–5) which had not been injected since March 29 and in cage 2, 5 individuals (6–10) which were still being injected. Injections were continued as usual with individuals 6–10 after the male had been placed in their cage. Of the 5 individuals (1–6) in cage 1, 3 bore young respectively on May 29, May 26, and May 31. No. 4 bore no young until July 8, and No. 5, no young at all. The inhibitory influence of the later injections on the individuals in cage 2 is indicated by the fact that no young were born in this cage before June 12 (individual 10). No 7 bore young June 19 and No. 9, on June 26. A male was placed in cage 3 with individuals 11–15 on May 31. No. 14 bore young on June 26. An inspection of Figure 1 shows that the dilutions used were obviously such as to produce only incomplete sterility since 8 of the 19 females eventually produced young, some of them (e.g., 1, 2, and 3) which had had a limited treatment, shortly after the male was introduced.

TESTICULAR NUCLEOPROTEIN EXTRACTS AND THE OESTROUS CYCLE

It had been found by the senior author that sterility could be maintained through injections of testicular nucleoprotein extracts.

It was considered desirable, therefore, to learn just what effects such injections have on the oestrous cycle and whether or not coincident structural changes can be detected in the ovary and possibly also in the anterior lobe of the pituitary body.

The graphs shown in Figure 2 depict the oestrous rhythm revealed through daily smears in 8 untreated females used as controls; those shown in Figure 3 present the oestrous histories of 17 females given intraperitoneal injections of testicular nucleoproteins of the bull.

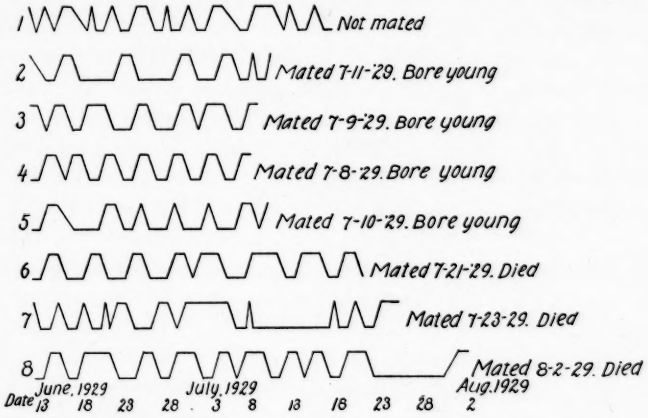

FIG. 2.—Graphs representing the oestrous cycles, as indicated by daily smears in 8 normal, untreated female rats.

The upper level of each graph indicates oestrum, the lower level, dioestrum; the lateral spread indicates time in days. The dosages varied in different rats as indicated in the graphs, the quantities in cc. being that amount of a 1 gram solution or suspension of dried nucleoprotein in 20 cc. of normal salt solution. Males were put into the cages about 2 weeks after the daily injections into the females were started, and they were kept under observation to see when the first copulations occurred. They were not regarded as "mated," as indicated in the graphs, until spermatozoa were recovered from the vaginal tract.

Of the females injected with solutions of nucleoproteins (Fig. 3), individuals 1–12 inclusive were given three injections at the time and in the quantities indicated on the respective graphs. With individ-

uals 13–16, inclusive, injections were begun at the point indicated by the arrowhead pointing toward the left and repeated daily to the point indicated by the arrowhead pointing toward the right. Of the

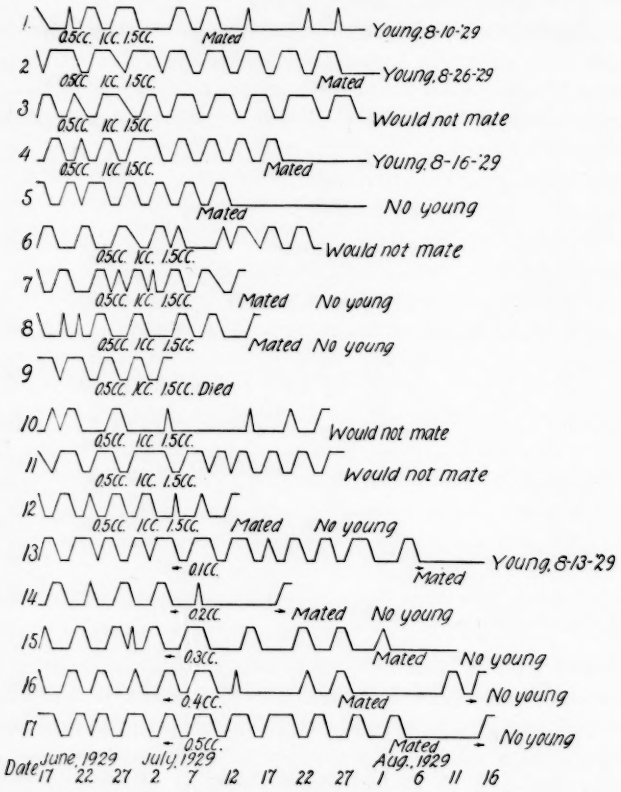

FIG. 3.—Graphs showing histories of the oestrous cycles of 17 rats injected with testicular nucleoprotein.

17 injected individuals, although all were mated or given opportunity to mate, only 4 (Nos. 1, 2, 4, 13) bore young. Four (Nos. 3, 6, 10, 11), although tested repeatedly, would not mate. The remainder were observed to mate and living spermatozoa were recovered from their vaginal tracts but they remained sterile until the discontinu-

ance of observation some months later. In several instances it will be noted that mating was followed by a prolonged dioestrum. This suggests the period of pseudopregnancy of 10–12 days which often follows copulation or even mechanical stimulation of the cervix uteri at certain stages in the oestrous cycle, as described by various observers (Long and Evans, 1922; Slonaker, 1929; Meyer, Leonard, and Hisaw, 1929).

It was decided to run still another series of sterility experiments with testicular nucleoprotein extract, using a larger number of rats and a more dilute dosage. It was also desired to see whether or not the rats could be held out of oestrus by increasing the dose when they showed evidence of resuming their regular cycles. The results are shown in the form of graphs in Figures 4 (controls) and 5*a*, *b* (experimental animals). The first injections consisted each of 0.5 cc. of a 1-gram solution of dried testicular nucleoprotein from the bull in 40 cc. of normal salt solution. When an animal came into heat, the dosage was increased to 1.0 cc.

The graphs shown in Figures 4 and 5*a*, *b*, record the histories of the oestrous cycles of 17 uninjected females used as controls and of 50 females injected with the nucleoprotein extract. A smear was made daily from each female and the stage in the oestrous cycle of each as thus revealed through microscopic examination was recorded in tabular form. The graphs in question were prepared from these tables. The upper level indicates oestrum, the lower, dioestrum; the lateral spread indicates time in days. A glance at Figure 4, which represents the record of the uninjected controls, shows a fairly regular oestrous cycle in most, with, however, an occasional prolonged dioestrum (10 or more days) in some, such as individuals 2, 10, 11, 12, 13.

In the graphs representing the injected females (Fig. 5*a*, *b*) the time at which the first injection was made is indicated by an arrowhead pointing to the left. In many instances this was at about the twenty-eighth day after the first making of smears was begun. The period between the beginning of the graph and this arrowhead, therefore, records the histories of the cycles of each individual prior to the first injections. The first injections were each always 0.5 cc. of a 1-gram solution of dried nucleoprotein in 40 cc. of normal salt

solution. Such intraperitoneal injections were made daily until the animal showed evidence of returning to the normal cycle, when the dosage was increased to 1.0 cc. to see if dioestrum could thus be

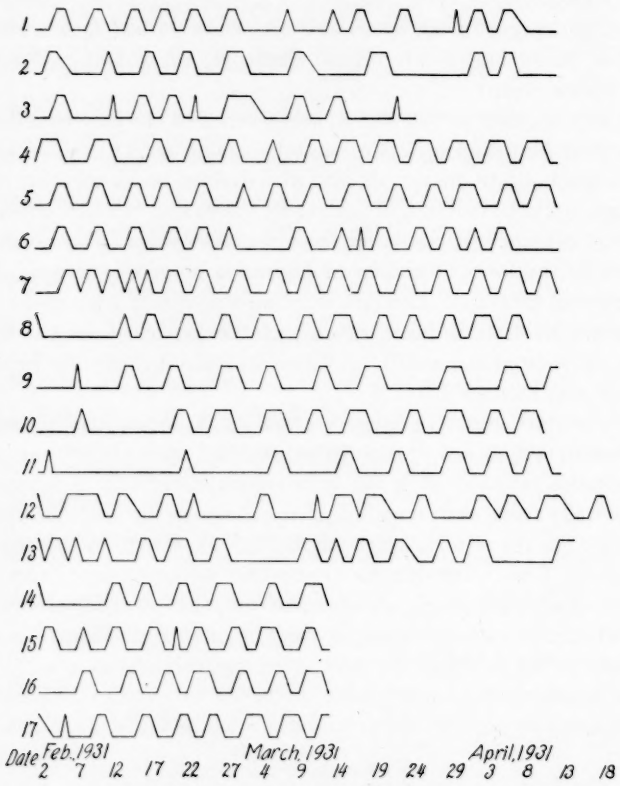

FIG. 4.—Graphs representing the oestrous cycles, as indicated by daily smears, in 17 normal rats used as controls in the experiment depicted in Figs. 5*a* and 5*b*.

prolonged. The time of such increased dosage is indicated in the graphs by an arrowhead pointing to the right. A glance at Figure 5 shows that the cycle was generally rendered irregular with prolonged dioestrous periods, although there were frequent temporary lapses into oestrum. The same indications of a pseudopregnant pe-

riod as suggested in connection with Figure 2 frequently followed shortly after the first injection and again after the first increased dosage.

The general impression which one gained in actual work with the animals was that a certain dosage would hold them out of oestrum for a time but that eventually this had to be increased to maintain a dioestrous condition. Any considerable increase in dosage, however, soon resulted in a condition of toxicity which the animal could not endure. In general, normal fertility seemed to return in about 6 weeks after the cessation of injections.

MODIFICATIONS IN THE OVARY AND PITUITARY ANTERIOR LOBE FOLLOWING INJECTIONS OF NUCLEOPROTEINS

Because of the known interrelations of gonads and pituitary it was obviously desirable to see what effect, if any, such a sterilizing solution as that of the testicular nucleoprotein was having on the ovaries or the pituitary, or possibly on both.

Sections of the ovaries of rats which had been subjected long to injection of testicular nucleoprotein usually revealed some increase in corpora lutea, but the most conspicuous change in histological appearance was due to the presence of a number of large follicles containing unshed ova; in many of these, extensive follicular degeneration was in evidence (Figs. 8 and 10, Pl. I). The appearance was that of constant follicular development without the occurrence of ovulation. In the larger follicles the granulosa layer remained intact and showed no trace of the luteinization which characterized the mature follicles of females injected with spermatozoa, described later. Follicular atresia, in fact, seemed to be the rule. Not infrequently a number of blastomere-like cytoplasmic masses, some nucleated, some not, had replaced the original ovum within the follicle (Fig. 10, Pl. I), giving the appearance of premature cleavage in some cases, in others, of mere fragmentation.

With the normal ovarian functions thus upset it was not surprising to find structural modifications occurring in the anterior lobe of the pituitary body, in such experimental animals. A histological study of these sterile individuals showed a marked increase in the number of basophile cells, in comparison with the oxyphile and

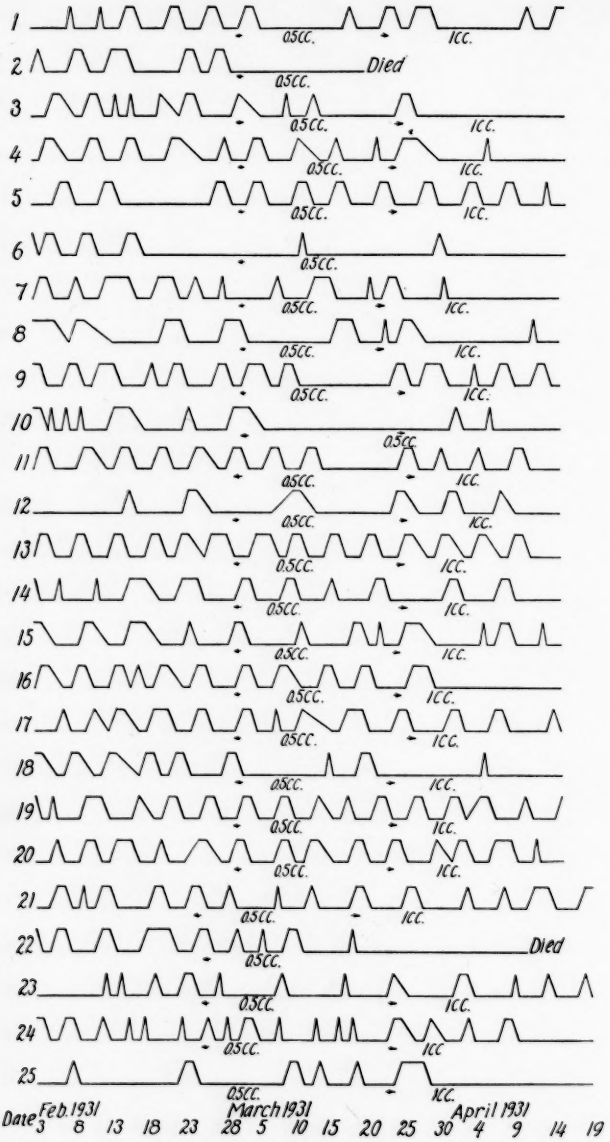

FIG. 5*a*.—Graphs showing (together with those in Fig. 5*b*) the histories of the oestrous cycles of 50 rats injected with testicular nucleoproteins. The data were obtained from the microscopic inspection of smears of the vaginal contents.

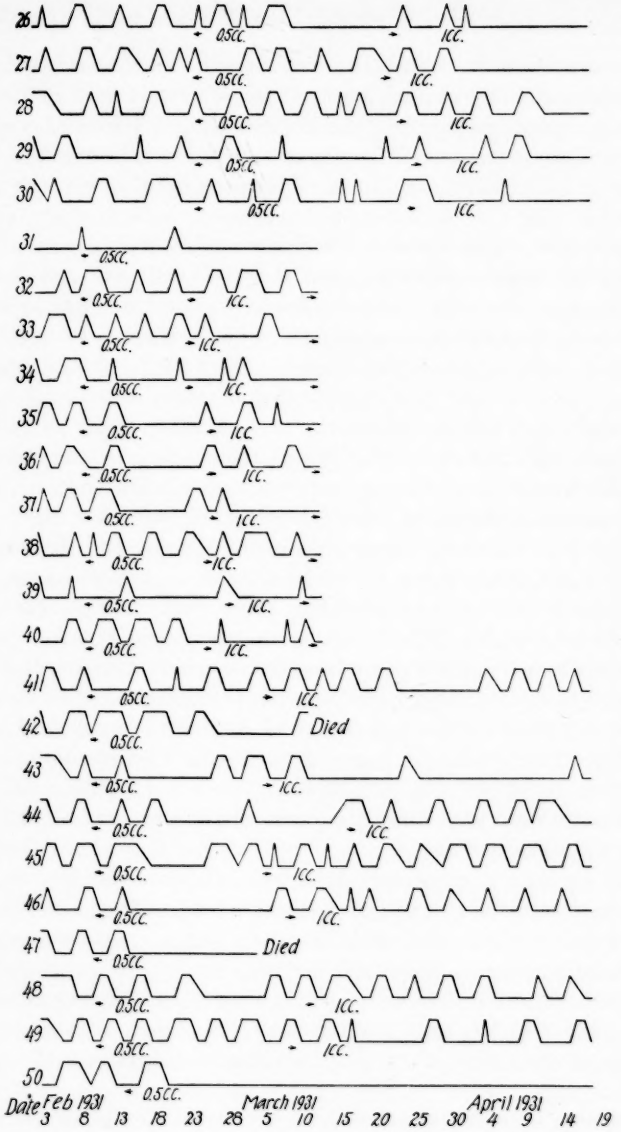

FIG. 5*b*.—See Fig. 5*a*

chromophobe cells. This is the typical picture of the anterior lobe of castrate animals (Addison, 1917). Figures 11, 12, 13, and 14, Plate II, show photographs from the anterior pituitary lobe of a normal rat, a castrate rat, and a female and a male rat subjected to injections of testicular nucleoprotein. The histological similarities in the castrate (Fig. 12) and the two nucleoprotein-treated individuals (Figs. 13 and 14) are evident; there is a marked increase in the number of the large coarsely granular basophile cells, and a scarcity of the type of cells with indefinite borders, known as chromophobes. In female, nucleoprotein-treated individuals, there is perhaps some slight increase in the oxyphiles—cells with denser-looking cytoplasm and affinity for acid dyes—although many sections show about the average number of the latter.

Figure 14, Plate II, is a photograph showing the histological structure of the pituitary anterior lobe in a male rat injected with a 1 to 20 solution of testicular nucleoprotein. The injections were given daily except Sundays, between October 30, 1931, and December 6, 1931. The section shows the same increase in basophiles as those from similarly treated females (Fig. 13) or from rats implanted with cancer (Fig. 18, Pl. III). In the larger basophiles (Fig. 14), the nuclei are often elongated and crowded to one side, apparently by the Golgi apparatus. Some nuclei were indented and looked much like those described by the authors (1932) in rats with cancer (Fig. 18, Pl. III). There is also, in contrast to the condition seen in the similarly treated females, a noticeable increase in oxyphile cells. This male was placed with 6 healthy females of known fertility and kept with them for upward of 5 months (December 6, 1931—April 25, 1932), yet none of the females bore young; hence either the male did not mate with them or the mating was infertile. The male was then killed and sections were made of testes, prostate, seminal vesicles, and adrenals, but no abnormalities could be detected in these parts. The testes showed the characteristic stages of spermatogenesis and the seminal vesicle and prostate both were lined with the typical columnar epithelium which characterizes the normal sexually active adult male.

The puzzling question of why the pituitary anterior lobes of males should respond differently to injections of nucleoprotein than those

of females remains unanswered so far. The authors have to content themselves with merely citing the fact. In the males, the decided increase in the number of the oxyphile cells is a result more like that following injections of spermatozoa, although in spermatotoxic males there is not the parallel increase in basophiles. The contrast in physiological effect between injected spermatozoa and testicular nucleoproteins, indeed, is in itself a surprising one, since the heads of spermatozoa are largely made up of nucleoproteins or substances which yield these upon chemical analysis. However, inasmuch as pure nucleoproteins supposedly do not produce antibodies and spermatozoa do, the difference in result, following injections, may be associated with this fact.

It seems not improbable that what exists in the female animals injected with nucleoprotein is essentially a castrate condition, the ovary being so affected by the treatment as to be functionally incapacitated. This would, in turn, account for the histological change observed in the anterior lobe. This conclusion cannot go unchallenged, however, since it is possible, of course, that the injected material acts directly upon both the ovary and the pituitary. In an earlier study (Guyer and Claus, 1932) we have shown that transplanted cancer, after about two months of growth, induces much the same histological picture in the anterior lobes of both males and females that castration does, yet in the case of the cancer the action seems to be directly upon the pituitary, inasmuch as no ovarian modifications were observable and the females continued their oestrous cycles and even bore young up until they were practically moribund.

In long-time castrates and in advanced cancer the basophiles not only increase in number and enlarge but eventually become greatly vacuolated (Figs. 17, 18, 19, Pl. III). In the nucleoprotein-injected animals we have observed little indication of the final vacuolated condition in the basophiles, but this is probably due to the fact that the increase in dosage necessitated from time to time renders the injected substance toxic and eventually lethal, before the basophiles have time to reach this final stage of vacuolization.

Inasmuch as Evans and Simpson (1929) showed that the gonad-stimulating power of the hypophysis increased after castration, and

PLATE I

FIG. 7.—Section (× about 19) of the ovary of a rat injected with spermatozoa. The lighter-staining regions in the walls of the follicles are occupied by lutein-like material (possibly correlated with increase of oxyphile cells in the pituitary, Fig. 16, Pl. III).

FIG. 8.—Section (× about 19) of the ovary of a nucleoprotein-injected rat showing scar-like remains of earlier follicles.

FIG. 9.—Section (× about 19) of the ovary of a normal, untreated rat.

FIG. 10.—A series of sections (× about 65) from ovaries of nucleoprotein-injected rats, showing young follicles containing fragmented ova. A normal ovum is seen to the left in the lower figure.

PLATE II

FIG. 11.—Section (× about 262) of pituitary anterior lobe of a normal, untreated rat. The darker, smal'er cells are the oxyphiles; the larger, lighter cells, the basophiles.

FIG. 12.—Section (× about 262) of pituitary anterior lobe of a recently castrated rat. The enlarged basophiles are evident.

FIG. 13.—Section (× about 262) of pituitary anterior lobe of nucleoprotein-injected female rat, showing increase and enlargement of basophile cells.

FIG. 14.—Less highly magnified section (× about 65) of pituitary anterior lobe of nucleoprotein-injected male, showing increase and enlargement of basophile cells (better seen in Fig. 15), and also increase in number of oxyphiles (rows of darker, smaller cells).

FIG. 15.—Section (× about 262) of pituitary anterior lobe of nucleoprotein-injected male showing increase and enlargement of basophile cells.

PLATE III

FIG. 16.—Section (× about 262) of pituitary anterior lobe of female rat injected with spermatozoa. The lobe is more highly vascularized (lighter areas) than in the normal pituitary, and there is an increase in oxyphile cells (darkly stained).

FIG. 17.—Section (× about 262) of pituitary anterior lobe of male, long-time, castrate rat. Basophile cells have increased in number and size and many of them are vacuolated.

FIGS. 18, 19.—Section (× about 262) of pituitary anterior lobe of rat to which cancer has been transplanted, showing increase in number and size of basophile cells. Some vacuoles are visible.

PLATE I

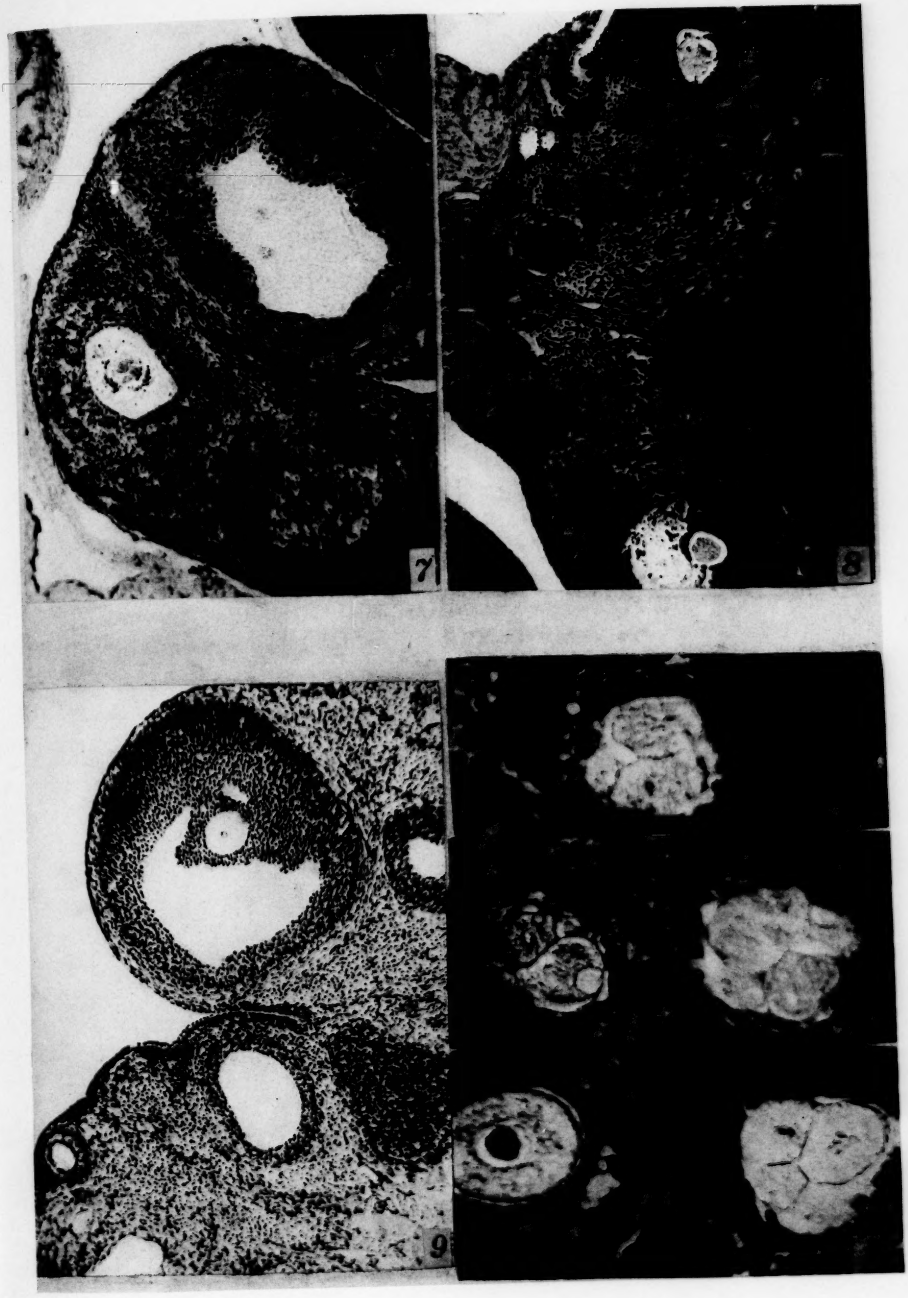

PLATE II

PLATE III

16

17

18

19

the present writers (Guyer and Claus, 1932) have shown that transplanted anterior-lobe substance of cancerous castrates is more potent in stimulating ovarian growth and sexual precocity than that of mere castrates, it was of interest to determine if a similar result would follow the transplantation of the pituitary anterior lobes of nucleoprotein-injected individuals, since they, too, showed a marked increase in the anterior-lobe basophile cells.

At the termination of the injection periods the pituitaries of injected and control animals were transplanted into young rats and mice of corresponding immaturity. The results are given in Table VI. It will be observed that in general a greater development of the ovaries followed implantations of the anterior pituitaries from the nucleoprotein-treated rats than from those of the normal controls, indicating increased potency of the former. Also, 20 male rats were castrated and 12 of them injected daily for 14 days with from 1.0 cc. to 1.5 cc. of the 1 to 40 nucleoprotein solution. The other 8 were kept as castrated controls. At the end of 14 days the pituitaries were transplanted into immature mice, all of corresponding immaturity, to test the relative potencies of the pituitaries of castrates compared with those of castrates which had also been injected with testicular nucleoprotein. The results are given in Table VII. The differences are too slight probably to be significant. While as they stand the data indicate that the anterior pituitaries of nucleoprotein-treated castrates are more powerful inducers of increased ovarian weight than are those of castrates alone, the number of animals used was obviously inadequate to render such a conclusion unquestionable.

SPERMATOTOXINS AND THE OESTROUS CYCLE

Inasmuch as the production of spermatotoxins by the injection of spermatozoa into females generally produces temporary sterility, and particularly since this seems to be the most effective way of inducing sterility by any means falling in the category of tissue-extracts or serums, it was thought advisable to study the effects of such injections on the oestrous cycle in greater detail than had heretofore been done. Although a former assistant (Pommerenke, 1928) of the senior author found that the injection of rabbit or rat spermatozoa or of fresh testicular extract into female rabbits had no effect

on the oestrous cycle as determined by breeding behavior and the familiar external evidences of oestrum, it was believed that a day by

TABLE VI

IMMATURE RAT

Donors	Number	Weight of Ovaries of Immature Rat at End of Experiment (In Grams)	
Four pituitaries from adult female rats injected with nucleo-protein, implanted in each immature rat	1	0.0127	
	2	0.0115	
	3	0.0092	
	4	0.0240	Av. 0.0149
	5	0.0172	
	6	0.0155	
	7	0.0144	
Four pituitaries from uninjected adult female rats, implanted in each immature rat	1	0.0101	
	2	0.0147	Av. 0.0116
	3	0.0100	

IMMATURE MOUSE

Donor	Number	Weight of Ovaries of Immature Mouse at End of Experiment (In Grams)	
One pituitary from adult female rat injected with nucleo-protein, implanted in each immature mouse	1	0.0027	
	2	0.0035	
	3	0.0056	
	4	0.0031	
	5	0.0034	
	6	0.0033	
	7	0.0048	
	8	0.0045	Av. 0.0043
	9	0.0081	
	10	0.0066	
	11	0.0037	
	12	0.0030	
	13	0.0037	
	14	0.0042	
One pituitary from uninjected adult female rat, implanted in each immature mouse...	1	0.0041	
	2	0.0043	
	3	0.0022	Av. 0.0036
	4	0.0031	
	5	0.0043	

day following of the cycle by means of the smear method would provide more accurate information on this point. Inasmuch as Pommerenke (1928) found that the longevity of spermatozoa normally

deposited in the genital tract of the female rabbit is greatly reduced by previous intravenous injections of rabbit spermatozoa, it was thought, furthermore, that by killing certain of the female rats at intervals after mating, one could determine whether or not the sperm were inactivated in the vagina, uterus, or oviduct. It was desired also to learn if such injections of spermatozoa caused any detectable histological changes in the ovaries or in the anterior lobe of the pitui-

TABLE VII

IMMATURE MOUSE

Donors	Number	Weight of Ovaries of Immature Mouse at End of Experiment (In Grams)	
One pituitary from adult castrate rat injected with nucleo-protein, implanted in each immature mouse	1	0.0073	
	2	0.0052	
	3	0.0052	Av. 0.0051
	4	0.0029	
	5	0.0053	
One pituitary from uninjected adult castrate rat, implanted in each immature mouse	1	0.0056	Av. 0.0048
	2	0.0040	

tary body. It is particularly important to know what other influences than the causing of temporary sterility spermatotoxins may be exerting, since certain advocates of birth control have suggested their employment for this purpose.

Sixteen young, sexually mature females were selected for intravenous injections with suspensions of bull spermatozoa (Fig. 6, Nos. 1–16) and 6 others (Nos. 17–22) were set aside as controls. The injections were given at intervals as indicated in the graphs of Figure 6. Usually 1–1.5 cc. of a 1 to 20 suspension of washed spermatozoa was used. Daily vaginal smears of each rat, experimental and control, were made.

In general, though various irregularities appeared in the oestrous cycles from time to time, these are no greater in most cases than those appearing in the normal rats (Nos. 17–22); hence we can only conclude that the cycle was but slightly if at all interfered with by the injections.

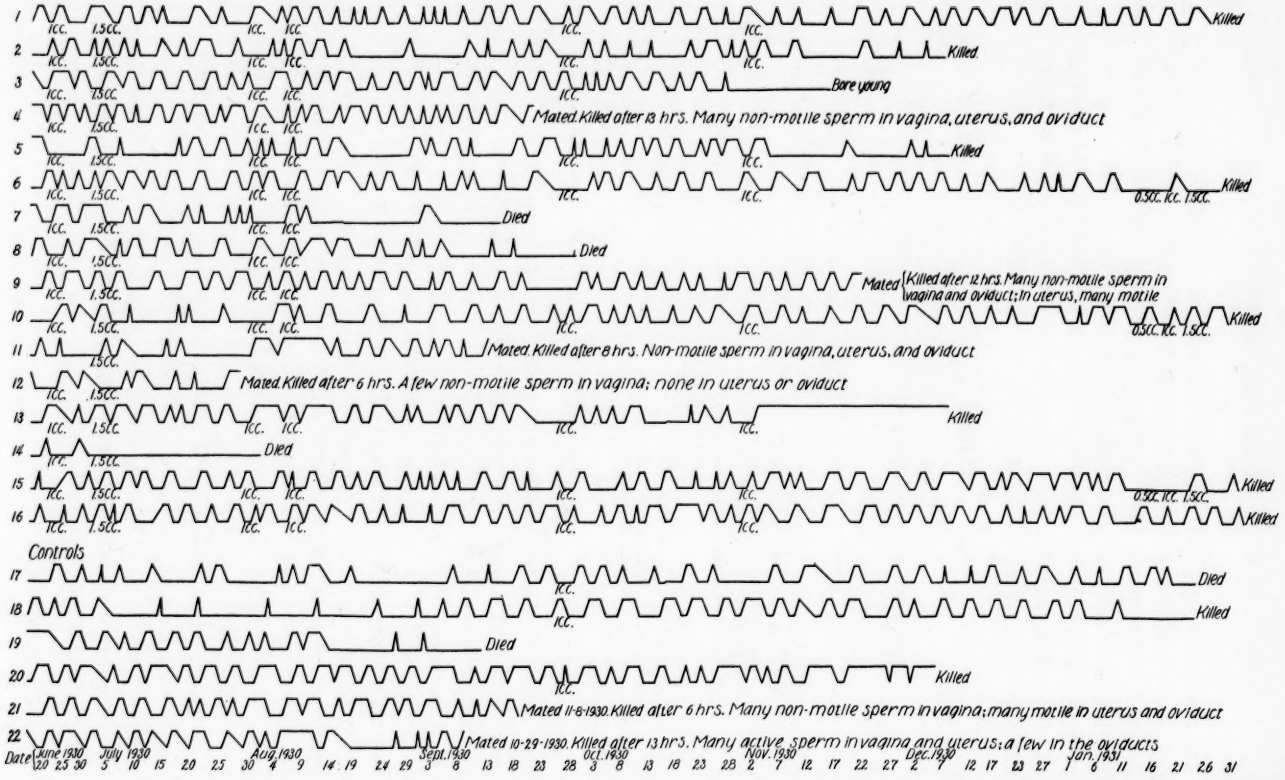
1 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. Killed
2 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. Killed
3 1cc. 1.5cc. 1cc. 1cc. 1cc. Bore young
4 1cc. 1.5cc. 1cc. 1cc. Mated. Killed after 13 hrs. Many non-motile sperm in vagina, uterus, and oviduct
5 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. Killed
6 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. 0.5cc. 1cc. 1.5cc. Killed
7 1cc. 1.5cc. 1cc. 1cc. Died
8 1cc. 1.5cc. 1cc. 1cc. Died
9 1cc. 1.5cc. 1cc. 1cc. Mated Killed after 12 hrs. Many non-motile sperm in vagina and oviduct; in uterus, many motile
10 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. 0.5cc. 1cc. 1.5cc. Killed
11 1.5cc. Mated. Killed after 8 hrs. Non-motile sperm in vagina, uterus, and oviduct
12 1cc. 1.5cc. Mated. Killed after 6 hrs. A few non-motile sperm in vagina; none in uterus or oviduct
13 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. Killed
14 1cc. 1.5cc. Died
15 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. 0.5cc. 1cc. 1.5cc. Killed
16 1cc. 1.5cc. 1cc. 1cc. 1cc. 1cc. Killed
Controls
17 1cc. Died
18 1cc. Killed
19 Died
20 1cc. Killed
21 Mated 11-8-1930. Killed after 6 hrs. Many non-motile sperm in vagina; many motile in uterus and oviduct
22 Mated 10-29-1930. Killed after 13 hrs. Many active sperm in vagina and uterus; a few in the oviducts
Date June 1930 20 25 30 July 1930 5 10 15 20 25 30 Aug. 1930 4 9 14 19 24 29 Sept. 1930 3 8 13 18 23 28 Oct. 1930 3 8 13 18 23 28 Nov. 1930 2 7 12 17 22 27 Dec. 1930 2 7 12 17 23 27 Jan. 1931 1 6 11 16 21 26 31

FIG. 6.—Graphs showing the histories of the oestrous cycles of 16 rats injected with spermatozoa, and of 6 normal controls

Following prolonged treatment, after July 8, 1930, the animals were placed with males every day, and if copulation occurred they were killed at varying periods and the viability of the spermatozoa recorded. The results are shown in Figure 6. The rats were given every known possible inducement to mate. For some reason most of the treated females were averse to mating and only 5 of them (Nos. 3, 4, 9, 11, and 12) would ever accept the males. One of these (No. 3) bore young, notwithstanding the spermatotoxin treatment. The other 4 were killed within 6–83 hours after copulation and the genital tract was examined for spermatozoa. In Nos. 4 and 11, spermatozoa were found in vagina, uterus, and oviducts, but all were dead, or at least non-motile. In No. 9, killed 12 hours after copulation, non-motile spermatozoa were found in oviduct and vagina, feebly motile ones in the uterus. In No. 12, killed relatively early in the experiment, about 6 hours after copulation, a few non-motile spermatozoa were found in the vagina, none in uterus or oviduct. In the controls (Nos. 21 and 22) which were killed after copulation—No. 21, 6 hours after; No. 22, 13 hours after—actively motile spermatozoa were found in the oviduct and uterus, although in No. 21 spermatozoa found in the vagina were non-motile.

From the foregoing results it appears that the sterility induced by the development of spermatotoxins in the female is not the result of any interference with the oestrous cycle but is probably due to immobilization of the spermatozoa in the genital tract, although imprisonment of the ova through precocious development of luteal substance, as discussed later, may also be a factor.

As to why so many of the treated females (Nos. 1, 2, 5, 6, 10, 13, 15, and 16) refused to copulate although coming into oestrum from time to time, the reason is not apparent unless the condition of the anterior pituitary, described later, may be a factor. In No. 2, which was running mostly "whites" toward the close of the experiment, the ovaries were unusually small, with but few corpora lutea; the uterus also was small but this would be expected in dioestrum. On the other hand, No. 5 had large ovaries with an abundance of corpora lutea which appeared normal; however, the uterus was small. No. 13 came into what was a perpetual oestrum, judging from the scales in the vagina, but she would not copulate. An autopsy showed her ovaries to be small with no large follicles; the uterus was of medium size, and

not large as would be expected of an animal in oestrum. One is inclined at first to attribute the disinclination of the sperm-injected females to mate to the effects of the spermatotoxins, but this is rendered somewhat doubtful by the fact that two (Nos. 18 and 20) of the controls showed a similar aversion to copulation. One of these, No. 20, when killed, was found to have large ovaries with large follicles and an enlarged uterus—the normal conditions for copulation—but still she would not mate.

Histological examination of the ovaries (Fig. 7, Pl. I) of such spermatotoxic rats has usually revealed not only traces of earlier corpora lutea but considerable numbers of medium- to large-size follicles which contain ova that seemingly are being retained unduly. Associated with this apparent inability to discharge the ova is a peculiar though not marked change in the follicular wall strongly suggestive of luteinization (Fig. 7, Pl. I). This fact coupled with that of an increase in the oxyphile cells of the pituitary anterior lobe, as described in the next paragraph, makes it seem not improbable that true corpus luteum substance is being formed in these follicles, since the studies of such workers as Charipper and Taylor (1931) indicate that increase in production of luteinizing hormone is correlated with increase in anterior pituitary oxyphile cells. The young growing ova of the sperm-treated rats, however, seem to differ in no way from those of normal individuals.

Sections of the pituitary anterior lobe of such sperm-injected rats (Fig. 16, Pl. III) present a different picture from that of the nucleoprotein-treated individuals (Figs. 13, 15, Pl. II). Instead of the marked increase of basophile cells observed in the latter, the number and size of the basophiles apparently remain normal. The oxyphile cells, however, are noticeably more abundant than in the average hypophysis. There is no observable difference in the chromophobe cells. It may be that this increase in oxyphiles lies back of the disinclination of the spermatotoxic rats to copulate. The same condition may apply to the male rat described under the discussion on the effects of nucleoprotein injections where likewise there was an increase in the number of oxyphile cells (Fig. 14, Pl. II). Apparently the oxyphile cells constitute the so-called "pregnancy" cells of various authors, in so far as we can interpret their descriptions, and these are supposed by some to release a luteinizing substance into

the blood-stream. It may be that in our sperm-injected animals an increased amount of such material is being released from the pituitary anterior lobe, which, although insufficient to suppress the oestrous rhythm as manifested in the vaginal epithelium, is potent enough to inhibit normal mating reactions, and to bring about precocious luteinization in follicles containing mature ova.

SUMMARY

1. Three successive hypodermic injections of an emulsion of combined epididymis and testis material of the rat, at intervals of 4 days, was ineffective in producing any appreciable sterility in rats.

2. Similarly, four injections of mammary substance at 4-day intervals produced no observable effect on fertility or oestrum.

3. Neither male nor female rats, subjected daily to ½-hour exposure to infra-red rays which raised the rectal temperature of rabbits 2°–3°, were rendered infertile, although 21 male rabbits of tested fertility, when similarly rayed on 10 successive days, were rendered sterile for an average of 77.5 days (Table I), leaving out of consideration 4 animals which became permanently sterile and 2 which died. Male rabbits seem particularly disposed toward infertility following physiological upsets such as those due to eye-injury or the injection of vaccines.

4. Seven injections of an emulsion of desiccated bull epididymis filled with spermatozoa, when injected into 20 female rats (Table II) at 5-day intervals, retarded fertility decidedly in most of the females and 6 remained sterile 6 months after the last injection—at the conclusion of the experiment. The litters of those which resumed fertility were reduced in numbers. Of 10 controls similarly injected with an emulsion of bovine mammary gland, 8 proved normally fertile, 1 died, and 1 was sterile.

5. In 18 female rats, each injected six times intraperitoneally with washed bull spermatozoa at 5-day intervals, fertility was noticeably delayed and diminished (Table III). Six of the 16 females which survived the experiment were apparently rendered permanently sterile.

6. Forty-one female rats, given successive injections of bull spermatozoa to render them infertile, were fed on dried testicular material two or three times a week to determine whether or not sterility,

once it was induced by spermatotoxins, could be maintained by oral administration of material containing the antigen for such spermatotoxins. No appreciable prolongation of the sterile period was obtained (Table IV). Twenty-five control animals injected with extract of dried rabbit salivary gland had their fertility diminished little if at all by the injections.

7. Twenty female rats were injected with extract of dried anterior pituitary body (0.75 cc. of a dilution of 1 part of dried material to 25 parts of liquid) every second or third day. Males of tested fertility were introduced into the cages after the sixth injection. Of the 16 females which survived the 2 months of injection, 1 bore 3 young during the course of treatment, 3 seemed to have been rendered permanently sterile, 9 showed temporary sterility and reduced fertility later, and 3 were killed for histological examination. The ovaries of the latter contained more than the usual number of corpora lutea, in some of which unshed ova were discernible.

8. In 5 female rats given smaller doses, the anterior pituitary substance did not inhibit ovulation. Possibly the result was due to the presence of a separable substance which induces precocious sexual maturity and which in dilute dosages apparently prevails over a residual fraction that inhibits ovulation and induces excessive development of luteal substance.

9. The oestrous cycles of 19 female rats injected with anterior pituitary substance was followed by means of daily inspections of vaginal smears. The results (Fig. 1) show that the cycles become irregular with a tendency for the inter-oestrual period to be much prolonged. Inhibition of fertility occurred as usual.

10. Of 17 female rats given intraperitoneal injections of testicular nucleoproteins, 4 eventually bore young and 4 would not mate; the remaining 9 copulated, but although living spermatozoa were recovered from their vaginas the individuals remained sterile until discontinuance of the experiment some months later. In several of the latter, prolonged dioestrum followed mating but whether this was a pseudopregnancy induced by copulation or the result of chemical inhibition is uncertain.

11. Another series of sterility experiments, with lighter dosage of testicular nucleoprotein extract, using 50 experimental rats and 17 controls, was conducted. The oestrous cycle was followed by means

of daily smears, and when the rats showed evidence of resuming their regular cycles, the dose was increased. The results (Figs. 5*a*, 5*b*) show that the cycle was generally rendered irregular with prolonged dioestrual periods. In most animals fertility returned in about 6 weeks after the cessation of injections.

12. Sections of the ovaries of rats which had been long subjected to injections of nucleoprotein usually revealed an increase in corpora lutea and in the number of follicles containing unshed ova. In some follicles ova were in the process of disintegration; in others, a fragmentation that suggested precocious cleavage had occurred.

13. Histological study of the pituitary anterior lobe in female rats rendered sterile by nucleoprotein injections showed a marked increase in the number of basophile cells in comparison with the oxyphile and chromophobe cells. The condition resembled that seen in castrate animals or in those to which cancer has been transplanted. In a male similarly injected there was also a noticeable increase in oxyphile cells, but in females such an increase occurred but slightly if at all.

14. Pituitaries of nucleoprotein-injected individuals were more potent than normal pituitaries in inducing precocious sexual development when transplanted into immature rats and mice.

15. The oestrous cycles of 16 rats kept sterile through injections of spermatozoa were followed by means of daily vaginal smears. The cycles (Fig. 6) were interfered with slightly if at all by the injections. The sterility induced by the development of spermatotoxins in females may be due to the immobilization of the spermatozoa which occurs in the genital tract.

16. Histological examination of the ovaries of such sperm-injected rats revealed many mature unshed ova with evidence of lutein-like material in the walls of the follicles. This may also be a factor in the sterility of these rats.

17. Sections of the pituitary anterior lobe of rats, in which spermatotoxins have been developed, show an increase in oxyphile cells but no visible change in basophile cells—a contrast to the condition which follows injections of nucleoproteins. Possibly this increase is followed by the release of a secretion which induces precocious luteinization in the follicular walls and thus prevents shedding of the mature ova.

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RESPONSE TO LIGHT IN THE CERCARIAE OF BUCEPHALUS ELEGANS[1]

(Six figures)

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NUMEROUS investigators have studied the problems concerning responses to light in organisms such as orientation, aggregation, sensitization, relative effect of wave lengths and intensities, and the effect of sudden change of intensity.

Reactions which are dependent on sudden change of intensity have been found to occur in many organisms (Mast, 1911, pp. 246–51). Some organisms respond to increase, others to decrease, and still others to both increase and decrease, in light intensity.

If the change in intensity to which these organisms are subjected does not take place rapidly, a response usually does not occur. It is evident therefore that the reaction is dependent upon the rate of change in light intensity. These reactions are now generally known as "shock reactions." They are characterized by the fact that the energy necessary to induce a reaction must be increased or decreased at a certain rate. The minimum amount of change in energy necessary to induce a response is known as the threshold.

In many organisms it is not necessary to expose the receptors continuously in order to induce a response. There is a period during which they must be exposed to light, followed by a period during which exposure is not necessary. If, for example, photosensitive seedlings are exposed to a horizontal beam of light for a short time and then placed in darkness, the seedlings will bend in the same direction that they would have bent had they remained in the light continuously. The time during which the stimulating agent must act in order to induce a response has been designated stimulation

[1] Contribution from the Zoölogical Laboratory of the Johns Hopkins University, and the Mt. Desert Island Biological Laboratory. The writer is very much indebted to Professor H. T. Folger, who suggested the problem and directed some of the work, and to Professor S. O. Mast, for invaluable suggestions and criticism made during the course of the experimental observations and the preparation of the manuscript.

period, exposure period, sensitization period, and presentation time; and the period during which exposure is not necessary, latent period. The sum of these two periods is known as the reaction-time. The time which elapses from the application of the stimulus until the organism resumes its former physiological state has been designated the recovery period.

The relations which exist between the stimulating agent and the various phases of response have been investigated in many organisms. The first attempt at a quantitative analysis of the response to light was made independently by Blaauw (1908 and 1909) and Froeschel (1908). These authors exposed the plumules of seedlings of *Avena* in a horizontal beam of light and ascertained in different intensities the duration of the illumination required for 50 per cent of them to bend. They maintain that the time (t) varies inversely with the intensity (i), that the product of the intensity and the time is constant, and that their results are therefore in accord with the Bunsen-Roscoe law. Ewald (1914) maintains that in *Daphnia* the amount of light energy required to evoke a response is constant over a large range of intensities. Hecht (1918, 1919*a*) maintains that it holds for *Ciona* and *Mya*, and Obreshkove (1921) concludes that it holds for the tadpoles of *Rana* under certain conditions.

Arisz (1911) contends that in *Avena* there is no definite threshold, that is, that, no matter how small the amount of energy is, it acts on the plant and is expressed by an effect, and that it is impossible to ascertain when a response begins. Folger (1925) maintains that in *Amoeba proteus* the amount of light energy required to induce a response varies with the intensity. Pieron (1926) repeated the work of Hecht on *Mya* and concluded that higher intensities are more efficient in inducing a response than lower intensities, i.e., that the Bunsen-Roscoe law does not hold. Folger (1926) also repeated the work of Hecht and his results confirm Pieron's contentions. Burkhardt (1926) repeated the observations of Blaauw and Froeschel on the seedlings of *Avena*, and the results which he obtained do not confirm their conclusions that the Bunsen-Roscoe law holds.

From the preceding account it is evident that in regard to the relation between intensity of illumination and the stimulating effect of a given amount of light there are divergent views. Blaauw, Froe-

schel, Loeb, Hecht, and others maintain that, if the product of the intensity multiplied by the time is constant, the response is constant, no matter how the two factors vary, i.e., that the Bunsen-Roscoe law holds. Folger, Pieron, Burkhardt, and others maintain that the stimulating efficiency of light varies with the intensity, i.e., that the Bunsen-Roscoe law does not hold.

Mast (1923, p. 179) in a discussion concerning this law concludes that "there are no reactions to light which are not in accord with the Bunsen-Roscoe law in certain respects and that there are none which are in accord with this law in all respects," and that "the statement so frequently made without qualification that this or that reaction is in accord with the Bunsen-Roscoe law is meaningless."

The present investigation concerns the behavior of the cercariae of *Bucephalus elegans* with special reference to the quantitative relation between stimulation and response to light.

MATERIAL

The cercariae of *Bucephalus elegans* are immature trematodes found in the gonad region of the fresh-water mussel *Eurynia iris* (Lea). The mussels occur in shallow beds in the Huron River, near Ann Arbor, Michigan. During the course of this investigation several hundred specimens were collected in this region.

Only a small proportion of the mussels collected were parasitized. They were identified as follows: all the specimens were put into finger bowls, one in each, and kept at a temperature of slightly more than 20°. At this temperature infected mussels readily shed their cercariae. Frequent examinations were made over a period of several days to ascertain which specimens were parasitized.

Following the period of isolation, the parasitized specimens were put into cylindrical battery jars, one in each jar, which had been previously supplied with tap water and a rich supply of green organisms. This "set up" was found to be very efficacious, for mussels were kept alive and in good condition for more than eight months.

A heavily parasitized mussel, when brought into the laboratory at ordinary room temperature, often sheds as many as one hundred cercariae within 24 hours. This was usually undesirable, and in order to obviate this high rate of shedding, the jars containing the mussels

were kept at a temperature below 15°. This simple procedure had the desired effect of reducing the shedding rate to practically zero. By keeping the mussels at a low temperature, it was possible to conserve the supply of cercariae over a period of several months. Whenever cercariae were needed for experiment, one of the jars containing a parasitized mussel was brought into the laboratory and as soon as the contents of the jar reached a temperature of 20° cercariae usually began to emerge. It appears that the most important factor in shedding parasites by this mussel is the temperature.

LOCOMOTION

The cercariae of *Bucephalus elegans* are expelled through the exhalant siphon of the mussel and immediately following liberation they start swimming toward the surface of the water. The most striking feature in their locomotion is the enormous contraction and expansion of the tail-furcae (Fig. 1, *a*, *b*, *c*). The body undergoes an undulating movement, but in comparison with that of the furcae, this movement is negligible. In swimming, the body hangs downward, with the furcae extended outward and upward in the form of a sigmoid curve. By means of extension and contraction of the furcae, the cercariae move slowly and gracefully through the water, seemingly in any direction. In swimming, the long axis of the body usually assumes a vertical position.

METHODS

The experiments on the reactions to light in the cercariae of *Bucephalus elegans* were made in a basement dark room; observations were made both with and without the aid of magnification. The source of light used was a 115-volt, 1,000-watt stereopticon lamp, mounted in a light tight shaft which was constantly ventilated so as to avoid excessive rise in temperature. The lamp was connected with a direct-current lighting system and maintained at 110 volts by means of variable resistance. A horizontal beam of light from an aperture 3 cm. in diameter was transmitted directly to a vertical microscope stage on which an observation aquarium was held by means of a mechanical stage (Fig. 2). The observation aquarium used in these experiments was constructed by fastening, with De

Khotinsky's cement, modified glass depression cells to microscope slides. Part of the cell was cut away to make an opening for the introduction of the cercariae. The aquarium was U-shaped inside and measured 20×15×3 mm. (Fig. 3). Different intensities were secured by moving the microscope stand closer to, or farther from the light.

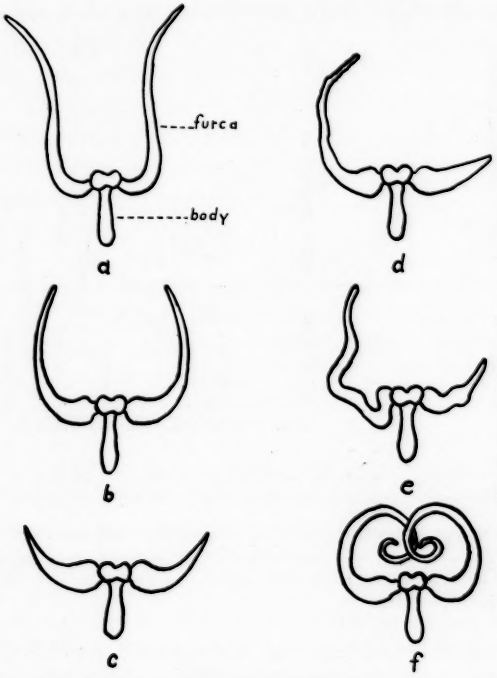

FIG. 1.—Outlines illustrating swimming movements of cercariae: *a*, *b*, *c*, normal movements; *d*, *e*, *f*, response to rapid increase in illumination.

A permanent scale in centimeters made by measuring the distance from the center of the lamp to the center of the observation aquarium on the microscope stage was marked off on a rack which supported the microscope stand. This made it possible to obtain any desired intensity rapidly and accurately. The light was turned on and off by means of a photographic shutter mounted in front of the

aperture in the wall of the ventilating shaft. The light passed through a cell 3 cm. thick containing distilled water interposed between the shutter and the lamp (Fig. 2).

RESPONSE TO RAPID INCREASE IN ILLUMINATION

When subjected to sudden increase in illumination cercariae usually respond with a slight shuddering movement, followed by an

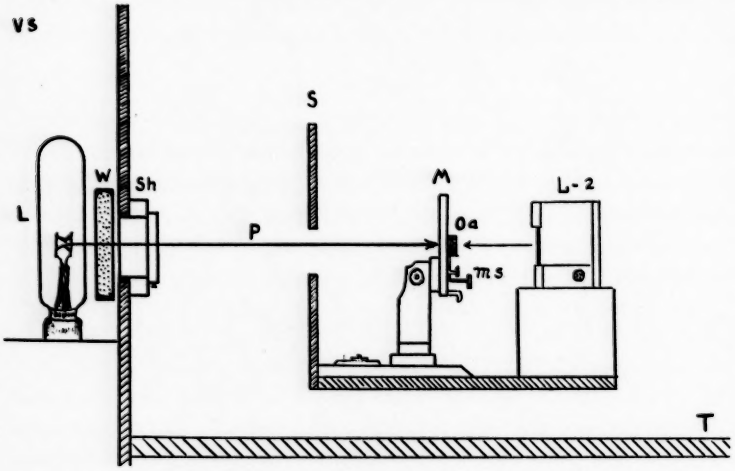

FIG. 2.—Diagram of apparatus used in studying the reactions of cercariae to light. *L*, 1,000 watt lamp, L_2, weak ruby lamp; *M*, microscope stand; *Oa*, observation aquarium; *P*, path of light; *S*, screen; *sh*, shutter; *ms*, mechanical stage; *T*, table; *vs*, ventilating shaft; *W*, distilled water.

inward coiling of the furcae (Fig. 1, *d e, f*), after which they slowly sink to the bottom of the aquarium. This reaction occurs only when the intensity is rapidly increased. If the intensity is gradually increased it does not occur. The cercariae recover either in light or darkness; recovery in darkness requires about one minute. In light it takes considerably longer. Recovery takes place as follows: at first the furcae slowly uncoil, then they undergo a shuddering movement, after which erratic expansion and contraction occur, and finally they swim normally again (Fig. 1, *f–a*).

Under favorable conditions the cessation of movement following

rapid increase in illumination is very marked and can be clearly seen. This made it possible to measure fairly accurately the time occupied by the different phases of response described below.

The observations on response to rapid increase in illumination were made as follows: a cercaria to be used for experiment was taken with a pipette, put into an observation aquarium in the dark room, and kept there for an hour or more to insure complete adaptation to the conditions of the room. A weak ruby light which did not appreciably affect the cercariae was now turned on, after which the aquarium containing the adapted cercaria was fastened to the mechanical stage on the microscope, and the stage placed at the desired distance from the lamp.[2] Then by opening the shutter the strong light from the projection lamp was suddenly flashed on the cercaria and left until a reaction occurred, after which the shutter was closed and left for 3 minutes and then opened again. The reaction-time was measured with a split-second stop watch. The number of times a specimen responded in succession varied somewhat, depending on its physiological condition. Some of the cercariae ceased to react after one or two exposures, others responded more than sixty times at 3-minute intervals with a maximum variation of only 5 per cent in the reaction-time.

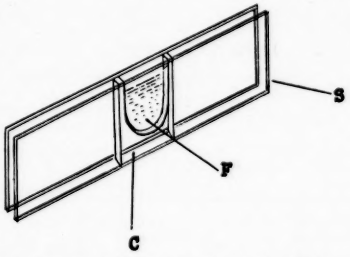

FIG. 3.—Sketch showing construction of observation aquarium. *C*, cell; *F*, fluid, *S*, microscope slide.

RELATION BETWEEN REACTION-TIME AND LUMINOUS INTENSITY

Table I contains the reaction-time obtained as described above for several specimens each subjected to light of diverse intensities. By referring to this table it will be seen that in every individual tested the reaction-time varied inversely with the luminous intensity,

[2] In measuring the reaction-time to light in the cercariae of *Bucephalus elegans*, precaution was always taken to illuminate the entire body and furcae of the specimens from the side; thus any possible effect due to differential sensitivity in the specimen was eliminated.

but that there was some individual variation; the reaction-time of different individuals in the same intensity varies from 8 to 12 seconds in the low intensities.

By referring to Figure 4 it will be seen that if the total average of the reaction-time obtained for all the individuals treated is plotted against the luminous intensity, a curve is produced which simulates a hyperbola. The relation between reaction-time and luminous intensity for the cercariae of *Bucephalus elegans* is in fairly close agree-

TABLE I

RELATION BETWEEN REACTION-TIME AND LUMINOUS INTENSITY

Intensity M.C.	Average Reaction-Time (Seconds) 3–5 Readings of Individuals *a–h*								Total Average
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	
16,800	3.5	2.2	4.5	2.6	3.4	4.0	3.2	2.6	2.9
12,640	4.1	2.6	4.6	3.7	3.9	.....	3.4	3.7	3.8
9,480	4.9	4.8	5.3	4.5	5.5	5.3	4.8	5.5	5.0
6,320	5.2	6.0	5.7	7.3	7.1	5.4	7.0	7.5	6.4
4,740	7.5	.....	8.3	.....	.....	8.4	.....	.....	8.0
3,160	8.4	11.7	10.3	13.5	8.6	.....	12.7	12.6	11.1
1,580	13.1	23.0	13.6	24.9	13.3	11.5	23.7	21.7	18.1

ment with that obtained by others for other organisms, e.g., that obtained by Blaauw for *Avena*, by Hecht for *Ciona* and *Mya*, and by Folger for *Amoeba*.

RELATION BETWEEN STIMULATION PERIOD, LATENT PERIOD, AND LUMINOUS INTENSITY

As previously stated it has been observed that the reaction-time is made up of two components: the stimulation period, the time during which it is necessary to expose the receptors; and the latent period, the time during which it is not necessary to expose the receptors in order to elicit a response.

The stimulation period was ascertained as follows: the reaction-time for a selected cercaria was ascertained for a given luminous intensity, then the cercaria was exposed successively for shorter and shorter periods of time with an interval of 3 minutes between the successive exposures, until no response occurred. The minimum

time during which the light must act to produce a response was thus ascertained; this is the stimulation period. This procedure was then repeated with 15 other individuals at diverse intensities. The results obtained are presented in Table II and Figure 5.

Examination of Table II shows that, in the specimens tested, the stimulation period varied inversely with the luminous intensity,

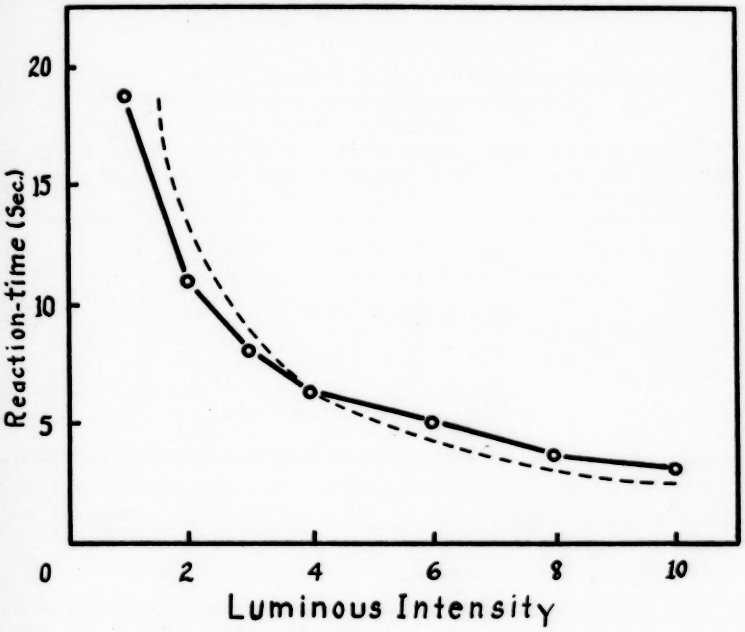

FIG. 4.—Relation between reaction-time and luminous intensity. Total average reaction-time for 8 specimens, 5 readings made on each specimen.

i.e., the more intense the light, the shorter the exposure required to obtain a response. It also shows that the latent period varied inversely with luminous intensity. It shows moreover that as the intensity decreased the energy required to induce a response rose to a maximum at 3,160 m.c. and then fell. This shows that the Bunsen-Roscoe law does not hold for the cercariae of *Bucephalus elegans* under the conditions of the experiment.

EFFECT OF TEMPERATURE ON PHOTIC REACTION-TIME

Several investigators maintain that temperature has an effect on photic response. De Vries (1913) maintains that in the seedlings of *Avena* the reaction-time depends upon the temperature. Hecht (1919*c*) comes to the same conclusion regarding *Mya*, and Cole (1922) contends that photic reaction-time in *Necturus* varies with the temperature. Crozier and Federighi (1924) maintain that increase in temperature accentuates photic circus movements in *Limax*. These investigators agree in that all maintain that increase in temperature results in decrease in the time required for various responses to light.

TABLE II

THE RELATION BETWEEN LUMINOUS INTENSITY, STIMULATION PERIOD, LATENT PERIOD, AND STIMULATION PERIOD TIMES LUMINOUS INTENSITY

Average in seconds of the results obtained by making 5–8 readings on each of 16 specimens. S.P., stimulation period; L.P., latent period; R.T., reaction-time; (it), intensity times stimulation period.

Intensity M.C.	S.P.	L.P.	R.T.	(it)
15,800	.8	3.2	4.0	12,640
12,640	1.1	3.1	4.4	13,904
8,480	1.9	3.7	5.6	16,112
6,320	2.9	3.8	6.7	18,328
3,160	5.6	4.7	10.3	18,696
1,580	6.7	5.8	12.5	10,586

MATERIAL AND METHODS

The apparatus used for regulating temperature consisted of a copper water bath having a circular opening $\frac{3}{4}$ inch in diameter in one side to admit light, a certified thermometer, and five 19-liter reservoirs fitted with glass and rubber tubing. By regulating the rate of flow of water through the water bath any desired temperature could quickly and accurately be obtained and maintained as long as desired.

The observations were made as follows: an observation aquarium containing a dark adapted cercaria was fastened with metal clips to the side of the water bath so that the center of the aquarium coincided with the center of the aperture. The water bath was so placed

that the light from the lamp passed directly through the center of the aquarium. The cercaria was then exposed to the light from the lamp until a reaction occurred, after which the shutter was closed and the cercaria left in darkness. During this interval the reaction-time was recorded, then the process was repeated five times with the same individual in the same intensity after which it was repeated with the

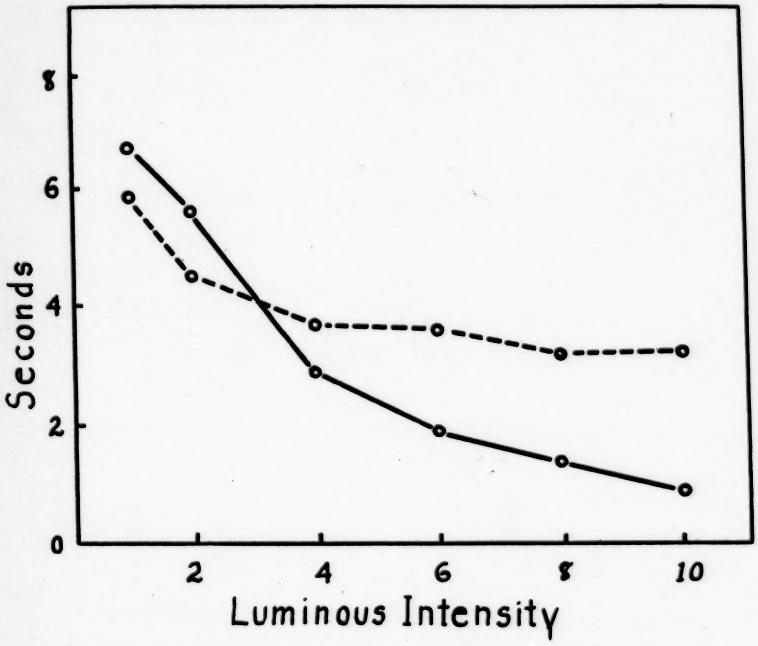

FIG. 5.—Relation between luminous intensity, stimulation period, and latent period. Curves represent the average results obtained in making 5–8 observations on each of 16 specimens. Solid line, stimulation period; broken line, latent period.

same individual in different temperatures allowing 10 minutes for adaptation at successive temperatures. This whole process was then repeated with each of 10 specimens.

RESULTS

The results obtained are given in Tables III and IV, and in Figure 6. Table III shows that the averages of the reaction-time for 5 indi-

viduals in 1,580 m.c. at temperatures 15°, 20°, and 25° were 15.1, 13.0, and 10.9 seconds, respectively, and in 9,480 m.c., 4.5, 3.9, and 2.7 seconds respectively. Table IV shows that the averages of the

TABLE III

RELATION BETWEEN TEMPERATURE AND PHOTIC REACTION-TIME
Each figure is the average of 5 readings.

Designation of Individuals	Average Reaction-Time (Seconds)					
	1,580 M.C.			9,480 M.C.		
	15°	20°	25°	15°	20°	25°
1	15.8	13.4	11.1	4.8	4.1	2.8
2	15.6	12.8	10.7	4.4	3.6	2.9
3	14.6	13.0	10.8	4.0	3.5	2.6
4	14.7	13.3	11.0	4.5	3.8	2.5
5	15.1	12.7	10.8	4.1	3.5	3.1
Total average	15.1	13.0	10.9	4.5	3.9	2.7

TABLE IV

RELATION BETWEEN TEMPERATURE AND PHOTIC REACTION-TIME IN 4,000 M.C.
Each figure is the average of 5 readings.

Designation of Individuals	Average Reaction-Time (Seconds) in 4,000 M.C.				
	10°	12.5°	15°	20°	25°
1	6.7	4.8	4.6	3.6	2.8
2	6.7	5.6	4.4	4.0	2.7
3	6.6	4.8	4.2	3.8	3.1
4	6.8	4.9	4.2	3.5	2.6
5	6.5	5.0	4.4	3.6	2.3
Total average	6.6	5.0	4.3	3.6	2.7

reaction-time for 5 other individuals in 4,000 m.c. at temperatures 10°, 12.5°, 15°, 20°, and 25° were 6.6, 5.0, 4.3, 3.6, and 2.7 seconds, respectively. This clearly demonstrates that increase in temperature caused a decrease in photic reaction-time over a wide range of intensities.

The relation between photic reaction-time and temperature is more clearly shown in Figure 6. This figure definitely indicates that the photic reaction-time varies inversely with the temperature, and

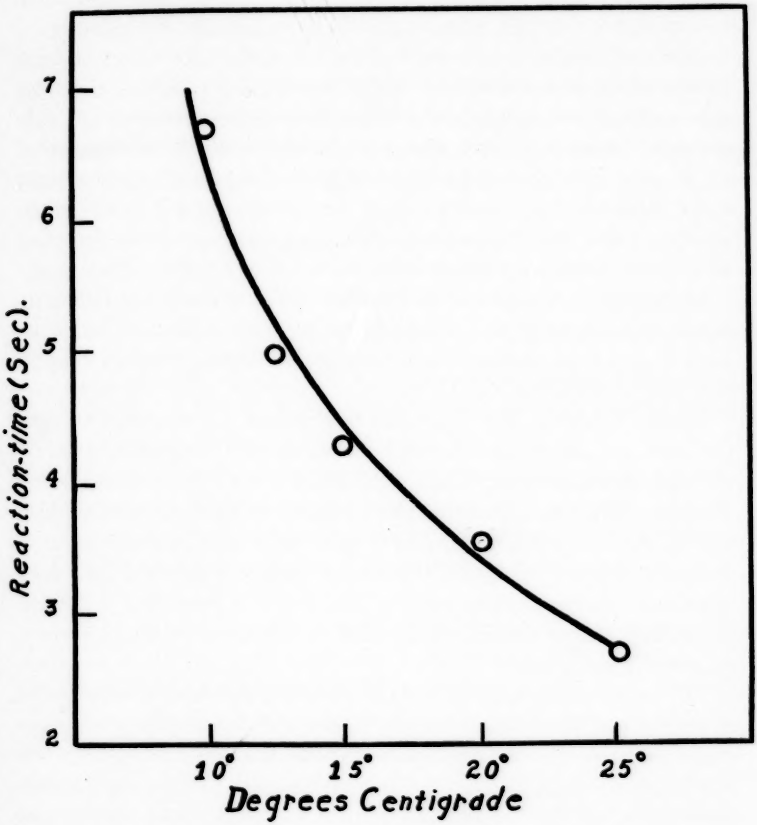

FIG. 6.—Relation between temperature and photic reaction-time in 4,000 m.c. Circles, experimental points; curve, a hyperbola (abscissae×ordinate=67).

that the relation between reaction-time and temperature follows the equation $xy=K$ when x equals the reaction-time, y, the temperature, and K, 67.

DISCUSSION

It has been held by some investigators that certain responses to light in organisms are correlated with photochemical reactions. Mast (1907, p. 159) postulated a reversible photochemical reaction to account for certain responses to light in *Volvox*. He assumes (1) that *Volvox* contains substances X and Y, the chemical reaction between which is regulated by the intensity of the light; (2) that a sub-optimum intensity or darkness favors the formation of substances represented by X and a supra-optimum, those represented by Y; and (3) that the colonies are neutral in reaction when there are Y substances in one member of the equation and X in the other; positive when one member contains $X+$ substances and the other $(Y-)$, and negative when one contains $(X-)$ and the other $(Y+)$.

He later (1927) modified this hypothesis by postulating two additional substances M and N which are non-photochemical but react with X and Y in such a way as to account for the effect of adaptation on the response.

Hecht (1918, 1919*a*, *b*, 1921), to account for the response to light in *Ciona* and *Mya*, postulated a reversible photochemical reaction like that postulated by Mast in his earlier work. He assumed that a substance S is changed under the influence of light to substances P and A, that a certain amount of S must be broken down in order to induce a response, that the stimulation period is the time necessary to convert the required amount of S to P and A, and that the latent period is the time during which other processes occur which are not dependent upon light.

The response to light of cercariae studied are fundamentally the same as those observed in *Volvox*, *Ciona*, and *Mya;* if they are associated with reversible photochemical reactions in the latter, one would expect them to be associated with similar reactions in the former. However, the response to light in the cercariae is also similar to the responses to light observed in *Amoeba;* and Folger (1925) contends that in *Amoeba* there is considerable evidence which indicates that photic stimulation is not photochemical. He maintains that mechanical shock produces cessation in streaming just as light does, and that, after a reaction to a mechanical shock, a period of recovery must be allowed before a specimen will respond to light again. He

concludes that "this seems to indicate that the processes involved in mechanical and photic stimulation are fundamentally the same . . . . , and it is evident, since mechanical stimulation is not photochemical, that photic stimulation cannot be photochemical."

In so far as can be ascertained, response in cercariae to mechanical and electrical stimulation is identical with that induced by increase in illumination. Therefore, if Folger's contentions are correct for *Amoeba*, it may be assumed that photic responses in cercariae are not correlated with photochemical reactions. It is evident from the above discussion that the results obtained do not clearly show the nature of response in cercariae. Some of the evidence seems to support the contention that responses are correlated with a photochemical process, while other evidence seems to support the contention that they are not.

SUMMARY

1. The cercariae of *Bucephalus elegans* respond to rapid increase in light intensity. This response is regular enough to be used in quantitative measurements.

2. Changes to which the cercariae of *Bucephalus elegans* are subjected must be fairly rapid in order to induce a response, in other words, the reaction is dependent on the rate of change in the magnitude of the stimulating agent. The response is therefore a "shock reaction."

3. When photic reaction-time is plotted against luminous intensity, the resulting curve simulates a hyperbola. The reaction-time therefore varies inversely with the intensity.

4. Reaction-time is made up of two components, viz., the stimulation period and the latent period. Both of these components vary with the intensity; the greater the intensity the shorter the stimulation period and the shorter the latent period. The maximum stimulation period and the maximum latent period occur at the same intensity, i.e., at the lowest intensity.

5. Stimulating efficiency varies with the luminous intensity; as the intensity increases, the amount of light energy necessary to induce a response gradually increases to a maximum, then gradually decreases to a minimum. This indicates that the Bunsen-Roscoe law

does not hold for cercariae under the conditions of the experiments made.

6. Some of the evidence presented seems to indicate that response to light in the cercariae of *Bucephalus elegans* is associated with a photochemical reaction. Other evidence lends support to the contention that it is not photochemical.

7. The effect of temperature on photic reaction-time is such that the reaction-time varies inversely with the temperature.

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GROWTH AND NUTRITION OF MELANOPLUS DIFFERENTIALIS THOMAS (ORTHOPTERA: ACRIDIDAE) I. GROWTH ON A SATISFACTORY MIXED DIET AND ON DIETS OF SINGLE FOOD PLANTS[1]

(Eight figures)

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INTRODUCTION

MELANOPLUS DIFFERENTIALIS is a convenient animal for biological study. It has been used by various workers in this laboratory for morphological, embryological, and physiological studies, and it has been raised upon mixtures of various leafy food plants. However, little attempt has been made to determine exactly its dietary requirements. The large number of widely different plants upon which it will feed, the general hardiness of the insect, and the ease with which it may be used—these indicate that it should be an excellent laboratory animal for the study of nutrition, in invertebrates at least; and that probably it will be valuable for the consideration of the general aspects of the study of nutrition as well. Though it must always be borne in mind that insects and the higher vertebrates are very dissimilar animals, it may well be that further investigation of the nutritional needs of the lower animals may throw light upon the general aspects of the study.

The work of Loeb and Northrop (1916) on *Drosophila*, showing the dependence of these flies upon yeast, led those writers to conclude that some insects at least need accessory food substances analogous to, though "specifically different from that needed for pigeons, rats, and other warm blooded animals" (p. 312, *loc. cit.*), Baumberger (1919), similarly, found *Drosophila* dependent upon

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yeast, but he considered the nucleo-proteins the important constituent.

Bacot and Harden (1922) reported that the two vitamins A and B, as understood in the study of vertebrate nutrition, are indispensable to *Drosophila* and that vitamin C is not necessary. In the nutrition of *Tribolium confusum* Duv. (*Coleoptera*), Sweetman and Palmer (1928) found a probable need for vitamin B, though no absolutely demonstrable need for vitamin A could be found. Richardson (1926) determined vitamin B to be necessary for the growth of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (*Lepidoptera*), and also a substance or substances "similar to or identical with vitamin A." Other writers, however, discussed by Sweetman and Palmer (1928), do not consider Richardson's findings in regard to vitamin A to be conclusive. Zabinski (1928) reported that the roach *Periplaneta orientalis* (*Orthoptera*) is unable to mature on synthetic diets without vitamins.

Many insects are intimately associated with micro-organisms of various kinds in various relationships; e.g., many termites, investigated by Cleveland (1928), exist in a relationship of obligatory symbiosis with their contained intestinal protozoa. Wiedemann (1930) finds similar dependence upon symbiotic micro-organisms among certain larvae of lamellicorn beetles. Such micro-organisms might readily provide accessory food factors. Attempts have been made to rear insects in aseptic conditions to eliminate such micro-organisms. Wollman (1922) reported the rearing of successive generations of flies (*Musca domestica* and *Calliphora vomitoria*) aseptically on sterilized media, suggesting that larvae of these insects either do not need vitamins required by vertebrates or else can synthesize their own from the substratum.

Other writers suggest that the need for vitamins may not be so urgent among insects generally as among vertebrates; or suggest, even, that insects do not need the vitamins that vertebrates find essential. Thus Baumberger (1919) concludes, from a consideration of the dietary needs of insects, that nitrogen is the most essential factor to be considered in their nutrition; and that in the case of such a form as *Drosophila*, for instance, the yeast plant is in the relationship of a "loose symbiosis" with the insect—the yeast elabo-

rating nucleo-proteins from urates, ammonia, and amino-acids. These nucleo-proteins then form a complete food for the *Drosophila*.

In regard to the various nutrients—aside from the dietary accessory factors or vitamins—the insects as a group show extremely variivariable needs. The wide range of food habits within this class is a matter of common knowledge. Quite obviously the insects of extremely unique food habits, such as the strictly xylophagous insects, for instance, could throw little light upon the general dietary needs of more omnivorous animals. And it is noteworthy, as Uvarov (1928*a*) points out, that the "apparent" food and "actual" food of insects must be carefully differentiated. Many materials "are bitten off" by insects "for reasons which have nothing to do with nutrition, e.g., burrows made in wood, or even in metal (lead, larvae of *Sirex*)." But there are insects of nearly the same food habits as many vertebrates. Such forms might prove valuable material for the study of the nutrition of animals in general.

Among the more omnivorous *Orthoptera*, the cockroaches have been investigated from the point of view of their amino-acid requirements by Zabinski (1929), who found *Periplaneta* unable to utilize glycocoll or gelatin, but thriving on ovalbumin, as sole source of amino-acid; unable to live when its entire nitrogenous supply was in the form of ammonium compounds; and two other roaches, *Blatta* and *Blatella*, unable to grow—though able to subsist—on a diet of pure carbohydrates.

References to the differential utilization of lipoids by an insect is made by Richardson (1926), who, working with the Mediterranean flour moth *Ephestia kuehniella* Zeller (*Lepidoptera*), found egg yolk or butter fat suitable for this insect, and the former the more readily utilized of the two, probably, he concludes, due to the greater abundance, or greater stability, of its included vitamin A content. But, on the whole, there is too little definite information available in the field of insect nutrition.

The preferences of the insect used in these experiments have been determined in the field, under natural conditions, to be *Ambrosia trifida* (tall ragweed), *Polygonum* sp. (smartweed); *Zea mays* (corn), the clovers, and other forage crops. In the laboratory it thrives on various mixtures of the leaves of lettuce, dandelion, grass, clover,

and wheat. It has been brought to sexual maturity on the leaves of plantain, dandelion, wheat or rye, alone; and mixtures of these and other leafy plants.

The object of this experiment was to make a preliminary study of the development of this insect on various plants that it will accept as food. This study will then serve as a basis for working out modified diets in the future. The ultimate aim will be to learn precisely the dietary requirements of this animal with reference to the nutrition of animals in general.

MATERIALS AND METHODS

Melanoplus differentialis may be obtained easily in the vicinity of Philadelphia and is of a convenient size for experimental use, the adults measuring 28–34 millimeters for the male, 34–44 milimeters for the female. These dimensions are attained in 40–60 days, depending on food and temperature. The species reproduces well in captivity. "A single female will produce three pods (of eggs) with a total of about 250 eggs" (Carothers, 1923).

The eggs are easily incubated in moist sand at a temperature, first, of about 25° C. for two or three weeks. Then the eggs should be placed at a temperature below 15° for a similar period to simulate normal winter conditions and to overcome a tendency to "hibernation" induced in many eggs by constant high temperatures, as recorded by Nelsen (1931). Finally the eggs may be transferred to the original higher temperature for a final week or two, when hatching should occur.

The newly hatched grasshoppers are about 5 millimeters in length. They are strongly positively phototropic and may be handled quite easily by taking advantage of that fact. They eat voraciously and can utilize many different plants as food, growing rapidly to the adult size, and being remarkably robust. *Melanoplus differentialis* is a relatively quiet, unexcitable grasshopper, a fact which facilitates its use in various kinds of apparatus and permits also easy handling, weighing, and transferring from one container to another.

Newly hatched grasshoppers, immediately after the "intermediate" or "embryonic" molt (see Nelsen, 1931, p. 478, or Uvarov, 1928*b*, p. 42) were weighed individually and placed in individual

cages, $8\frac{1}{2}\times7\times6$ inches, of the type described by Carothers (1923). Damp sand was kept in the bottom of each cage to a depth of about an inch to provide moisture and to facilitate cleaning. The cages were kept on shelves before a window of southern exposure in a room of average temperature of 28° C. The range of temperature was at all times within 25–32° C. except for a few hours on each of three days when it reached a maximum of 33°, and for a few hours, on each of three nights, when it reached a minimum of 21°. This range of temperature, though great, is believed not to have unduly affected the results of this study, as at all times individuals of different ancestry were kept together at these temperatures, on each of the diets used. Since considerable space was required by the experiment, an entire room, on the same general heating system as the laboratory building as a whole, was employed.

The animals on diets of single food plants were fed the leaves of barley, oats, rye, or wheat, grown in shallow metal trays in soil, and cut fresh daily. Others were fed the leaves of lettuce, also supplied daily, the outer green leaves only being used. Certain animals were fed a mixed diet of lettuce leaves, wheat leaves, and apple fruit.

The animals were weighed at definite times during the experiments, usually at 24- or 48-hour intervals, occasionally after greater lapses of time, and each individual was weighed at nearly the same hour of the day, every time.

RESULTS

One hundred and twenty-seven individuals contributed to the data used in this paper for deriving the individual growth curves expressing growth in weight. Fourteen of these received the mixed diet of lettuce and wheat leaves and apple fruit. Eighteen were fed barley leaves; 28, lettuce; 23, oat leaves; 19, rye leaves; and 25, wheat leaves; as shown in Table I.

The mortality among these animals, varying from 95.7 per cent on the diet of oat leaves to 17.4 per cent on the mixed diet, is in part explainable by general cultural conditions, caged existence, and perhaps some unavoidable mechanical injury. No animals were observed to be injured by the handling they received; but no doubt it was inevitable that daily or almost daily transfer of these small ani-

mals—too unintelligent to be readily "tamed" as the vertebrate laboratory animals may be—should have some deleterious effect on the weaker animals.

But the factors named were the same for all animals, regardless of diet; and it is noticeable that the percentage mortality is roughly in proportion to the nutritive value of the diets employed, as evidenced by growth in weight, regularity of development, and general vigor. The writer believes that he is quite justified in assuming that the two diets of lettuce and of oat leaves, respectively, are deficient.

TABLE I

NUMBER OF INSECTS USED IN EXPERIMENTS

Diet	Original Number	Transformed Number	Transformed (Per Cent)	Died (Per Cent)
Mixed	14	10	82.6	17.4
Wheat	25	18	72.0	28.0
Barley	18	8	44.4	55.6
Rye	19	8	42.1	57.9
Lettuce	28	6	21.4	78.6
Oats	23	1	4.3	95.7

On all of the diets except those of lettuce and oat leaves, the growth of the insects is remarkably similar. This is shown by the growth curves on Figures 1–6.

Melanoplus differentialis at hatching may weigh 4.0—7.5 milligrams, averaging 5.4 milligrams (data from 127 individuals). From this initial weight the insects grow rapidly to an average weight of 527.1 milligrams for the males (average of 31 individuals, all diets) or 831.6 milligrams for the females (average of 18 individuals, all diets) immediately after the final molt. In the adult stage thereafter, growth continues at a much slower rate for about two weeks till a maximum is reached. After this, there is an increasing loss of weight until death, in the case of the animals reared through the entire life-cycle without being mated. Mated animals show an irregularly fluctuating weight during the week or two of sexually active life, the weight increasing and decreasing with the development and discharge of the sexual elements.

As the nymphs seem most useful for physiological material and

have so far been more widely used than the adults, because of their smaller size, their inability to fly, and the greater certainty with which one can fix the biological age of the immature individuals, the animals in these experiments were most carefully observed up to the final molt; and data covering the pre-adult portion of the life-cycle, only, are presented in this paper.

A. INCREASE IN WEIGHT AS A CRITERION OF GROWTH

1. *The average growth curve, from data derived from any of the satisfactory diets.*—Each figure (1–6) represents an average curve of growth (bottom curve), derived from the data of the individual curves, smoothed by free-hand. This average curve is obtained by the method described by Calvert (1929) for the comparison of the development of different individuals, by "the plotting of definite fractions of the total growth" (percentages in this case) "against definite fractions of the time consumed in making that growth" (percentages of time used similarly) (p. 260, *loc. cit.*).

Examination of this curve shows it to be, through the greater part of its length, a typical S-shaped growth curve, as pictured by Titschak (1924), Przibram and Megusar(1912), and others, for various *Orthoptera*. The *Melanoplus differentialis* curve deviates in the latter 20–30 per cent (by time) from the truly typical S-form by a steeper slope and by the attainment of a peak of weight considerably greater than the weight at transformation to the adult state. This peak occurs usually in the last 5–10 per cent of the elapsed time of the pre-adult life-span and may achieve an excess over the weight just after the final molt of 222.7 milligrams, or 23.8 per cent for the females, or 132.2 milligrams, or 24.2 per cent for the males. This interesting peak of excess weight is shown for insects of two other orders (*Tribolium confusum* Duv.: *Coleoptera* and *Ephestia kuehniella* Zeller: *Lepidoptera*) by Brindley (1930). This peak, though destined to be lost in transformation, is typical of all normal *M. differentialis* curves, in a greater or less degree, and attains its maximum usually at 90–95 per cent of the elapsed time from hatching. Those few individuals whose curves do not show such a peak died within forty-eight hours or less after transformation in every case.

This curve may be considered as showing four separate portions of

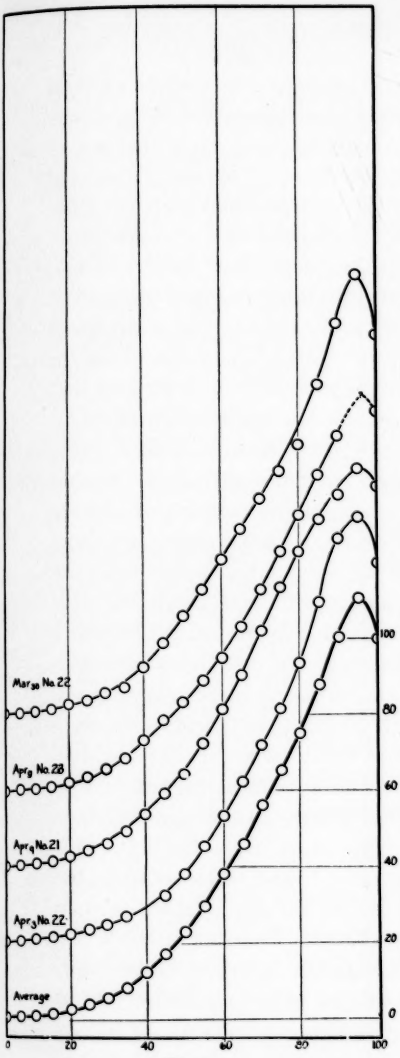

FIG. 1

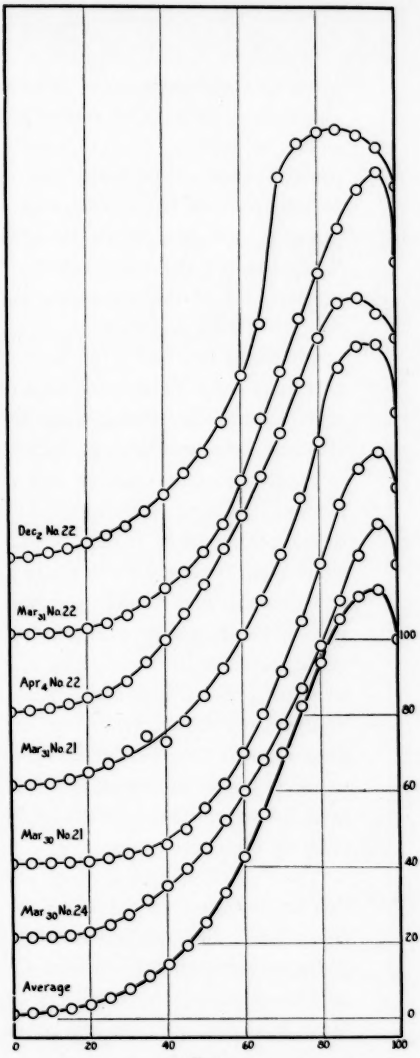

FIG. 2

FIG. 1.—Average and individual growth curves of females on the mixed diet of wheat and lettuce leaves and apple fruit. Individual insects indicated by date of hatching and a number. Abscissae—time, expressed in percentages of the time required for pre-adult life. Ordinates—weight, expressed by figures in right margin, in percentage of transforming weight; the figures referring to the average curve. NOTE.—each individual curve is displaced upward a distance corresponding to 20 per cent of transforming weight above the curve below.

FIG. 2.—Growth curves of males on the mixed diet. Ordinates and abscissae as in Figure 1.

strikingly different slope. The individual curves show these portions similarly. There is an initial portion wherein growth is slow, gradually increasing, which extends through approximately the first 13 per cent of the time consumed in pre-adult life. This merges into the second part of the curve, representing a more rapid increase, from about 13 per cent to 44 per cent of the elapsed time, in most cases. Then follows the most rapid rise of the curve, from 44 per cent to 90 per cent of the pre-adult life-span, reaching the decided peak in weight before mentioned. After this the rate of growth decreases throughout the last 5 per cent or 10 per cent of the pre-adult life to the final molt. This molt occurs usually between the fortieth and sixtieth days of the animals' lives, under the environmental conditions of temperature, etc., provided by these experiments.

Such smoothed curves are valuable in representing the general trend of growth, but do not, of course, tell the whole story of the development of an insect, which develops by larval stages or instars separated abruptly from each other by ecdyses and by more or less reorganization at each ecdysis. Yagi (1926) has shown that the growth of insects progresses as by "a series of S-shaped curves which correspond to the number of moults" (p. 2, *loc. cit.*) due to the stoppage of growth at each ecdysis, during a period of non-feeding and elimination of water. Except by very frequent weighings, this stoppage cannot be observed in the case of every molt of each individual of *Melanoplus differentialis*, but this loss of weight preparatory to some of the molts is shown by the data of some of the animals fed on each of the diets used.

The loss of weight is most considerable before the final molt, but can be demonstrated also in lesser degree before the other molts if the animal be weighed at the proper time. However, *Melanoplus differentialis* molts by preference at night or in the early morning, and frequently, therefore, twenty-four or occasionally forty-eight hours had elapsed between the last weighing and ecdysis. Of a total of 535 ecdyses especially noted for the purpose, 391 insects molted between the hours of 9 P.M. and 9 A.M.; and 144 between 9 A.M. and 9 P.M. In other words, 73.1 per cent molted at night or in the early morning. Therefore, not all curves can show the loss of weight at each ecdysis.

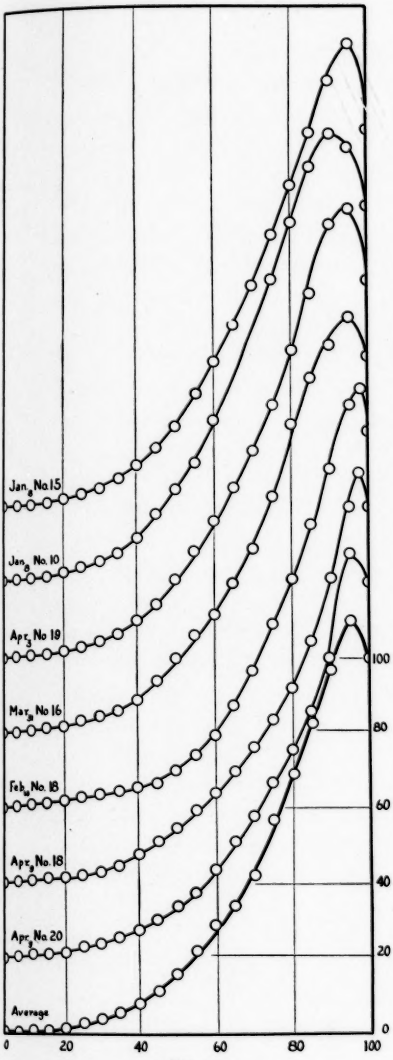

FIG. 3

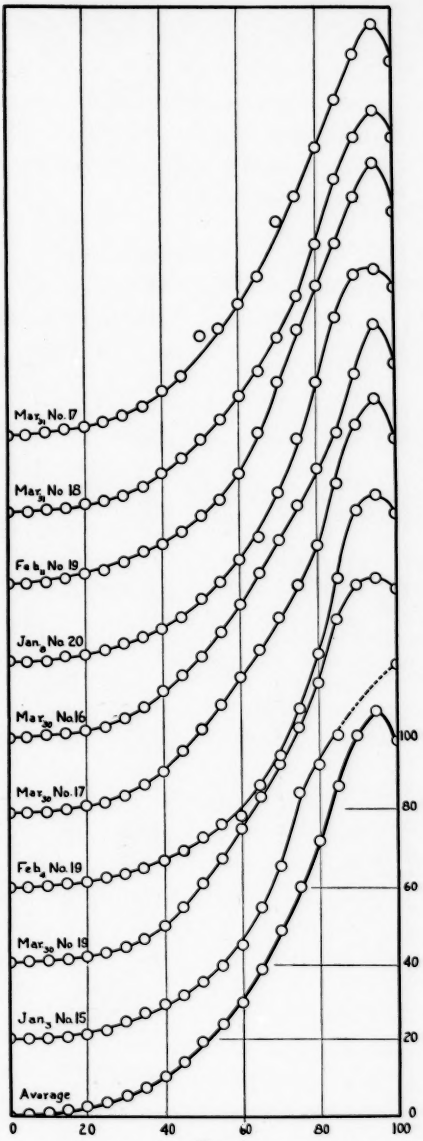

FIG. 4

FIG. 3.—Growth curves of females on the diet of wheat leaves alone. Ordinates and abscissae as in Figure 1.

FIG. 4.—Growth curves of males on the diets of wheat leaves alone. Ordinates and abscissae as in Figure 1.

Grasshoppers, like other insects, become quiet and sluggish preparatory to molting, and cease feeding for some time preparatory to the ecdysis itself. Uvarov (1928*b*) cites A. V. Valova as reporting that the Tryxaline grasshopper, *Chortippus scalaris* F.W., which exhibits four larval molts, fasts for an average period of 27 hours 20 minutes at the first molt, 32 hours 30 minutes at the second, 42 hours 40 minutes at the third, and 61 hours at the fourth. During this time, metabolism of necessity reduces the weight of the body, and it can be shown that the alimentary tract is quite emptied in preparation for ecdysis. In addition, large quantities of water are lost from the animal both before and during ecdysis. (One *Melanoplus*, for example, which molted in the weighing vial, weighed 40 milligrams before ecdysis. After molting, the animal weighed 37 milligrams and the exuvia, 0.8 milligram—a loss of 5 per cent of the animal's premolting weight in volatile materials, largely water—at ecdysis.) Exact measurements of the time during which *M. differentialis* takes no food have not been made, but the weight data would seem to indicate periods of time fairly comparable to those of *Chortippus*.

When the weight-growth curves—either the average or the individual curves—are further analyzed for the rate of growth, as in Figure 8, plotted by making a graph of the logarithm of the weight against percentage of the elapsed time of the pre-adult life, the resultant points fall into a series of straight lines of slightly differing slope. This indicates a slightly different rate of growth specific to each instar, except the last, which shows two or sometimes three rates. The first one or two of these different rates of growth in the sixth instar are increasing and lead up to the pre-adult peak of excess weight at about 90 per cent of the pre-adult life-span; after which the last instar is characterized by a decreasing rate of growth.

This is in agreement with the conclusion of Yagi (1926) that each of the instars of a developing insect represents a separate cycle of growth. It accords well, also, with the interpretation of growth suggested by L'Heritier and Teissier (1929) that "growth is at each instant characterized by a parameter of velocity which varies only by abrupt jumps. At the beginning of each cycle one can thus conceive a sort of impulse launching growth in a direction wherein it will

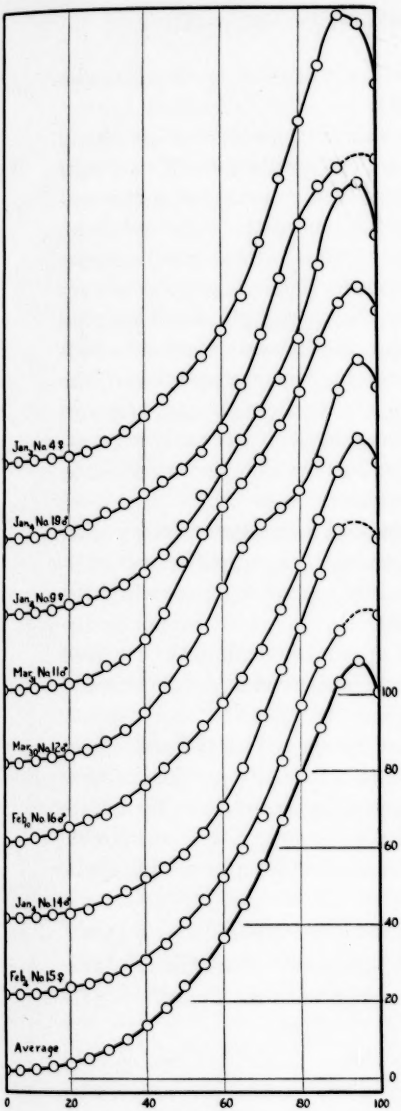

FIG. 5

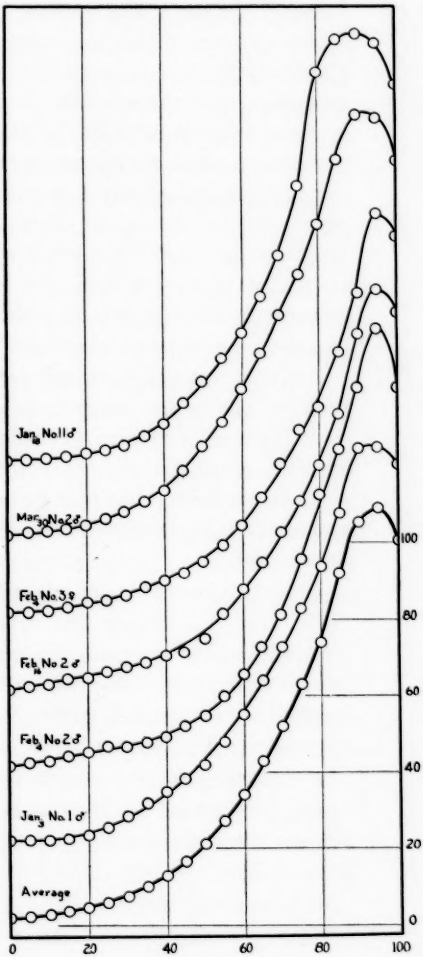

FIG. 6

FIG. 5.—Growth curves of insects on the diet of rye leaves alone. Ordinates and abscissae as in Figure 1.

FIG. 6.—Growth curves of insects on the diet of barley leaves alone. Ordinates and abscissae as in Figure 1.

persevere rigorously until there enters into play another impulse equally imperative" (p. 273, *loc. cit.*).

2. *Growth on the mixed diet of apple fruit and lettuce and wheat leaves.*—Figures 1 and 2 show individual weight-growth curves of females and males, respectively, on this diet, and average curves representing the data from all of each sex on this diet. These data were treated as were those for the average curve for all the insects, namely, percentage weight was plotted against percentage time, as suggested by Calvert (1929), as explained above (p. 332). In all essential respects these individual curves seem qualitatively similar to each other and to the curve representing the average condition. The length of pre-adult life varied from 57 to 83 days (average, 63 days for males, 72 for females) and the magnitude of the peak of excess pre-transforming weight is greater or less for different individuals, but the general trend of growth is similar.

Animals fed on this mixed diet suffered a smaller mortality than those on any other. They showed apparently a rather better condition of general nutrition, as evidenced by general vigor, ease in molting, long adult life, etc., but such criteria, obviously, cannot be applied in a strictly objective manner. Sexual maturity was attained and fertile eggs produced. This mixed diet is evidently sufficient and satisfactory.

3. *Growth on the diet of wheat leaves alone.*—Of all the single food diets used, this seemed the most satisfactory. Fewer insects were lost on this diet than on any other except the mixed diet. The adults reared through on this diet were vigorous, long-lived, and fertile. Typical weight-growth curves for females and males are shown in Figures 3 and 4, respectively.

4. *Growth on the diet of rye leaves alone.*—Rye proved nearly as satisfactory as wheat for *Melanoplus differentialis*. The animals thrive and are vigorous, the adults long-lived and fertile. Typical weight-growth curves are shown in Figure 5.

5. *Growth on the diet of barley leaves alone.*—Barley is about equally satisfactory with rye as a single food plant for this species. Sexual maturity was achieved and fertile eggs produced. Typical weight-growth curves are shown in Figure 6.

6. *Growth on the diet of lettuce leaves alone.*—Lettuce leaves alone proved unsatisfactory as the sole diet of this grasshopper, though in combination with other plants they are a very valuable adjunct to the diet. Mortality on this diet was very high (78.6 per cent of the animals failed to reach maturity). The adults did not live long, the majority dying within forty-eight hours after transformation. The apparently essential peak of excess weight prior to transformation was not put on by many of these insects. Their body tissues seemed softer, and less chitin seemed to be elaborated. These insects showed a condition that in vertebrate animals would be termed diarrhetic, and were, alone among the animals of these experiments, susceptible to some micro-organism in the rectum which caused considerable ulceration around the anus. The average weight-growth curve shows but a very slight pre-transformation peak of weight, since some individuals showed none at all. Some individuals showed a more typical curve, but growth was often irregular (Fig. 7).

7. *Growth on the diet of oat leaves alone.*—Oat leaves alone proved wholly unsatisfactory for this species, as previously reported by Carothers (1923). Only one individual was reared through to the adult state and it died the same day it transformed. The weight-growth curve for this animal is shown on Figure 7, below those fed on lettuce leaves alone. (This animal was not under constant observation by the writer, having been cared for during one week by the kindness of a colleague.)

The oat-fed animals of this species rarely survived the fourth instar, as had been reported for the species *Chortophaga viridifasciata* DeG. by Carothers (1923). The general vigor of the extremely young insects on this diet seemed unimpaired as compared with those on other diets. But they seemed, first, more excitable and, later, became more erratic in movement and less dependably phototropic than the other animals, finally growing successively weaker till they died. (In this connection an observation on a barley miner, *Hydrellia griseola* Fall.: *Diptera*, by Körting [1931] is interesting. This insect, though belonging in a different order, also does not thrive on oat leaves. It does not grow so large as on barley, grows more slowly, changes its mine often, and only 42 per cent of the larvae pupate.)

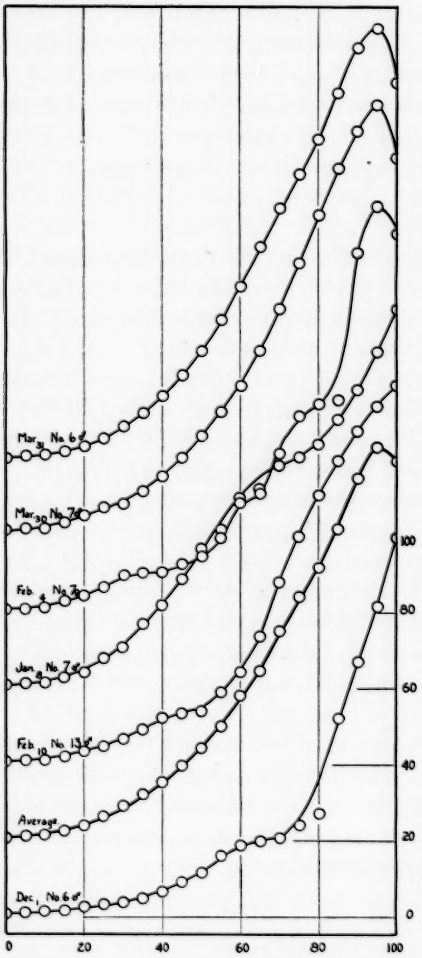

FIG. 7.—Growth curves of insects on the diet of lettuce leaves alone (upper 6 curves) and on oat leaves alone (bottom). Abscissae as in Figure 1. Ordinates as in Figure 1, the marginal figures in this case referring to the curve of the insect on the diet of oat leaves.

B. LARVAL INSTARS AS CRITERIA OF GROWTH

The instars or larval stages of insects have been widely used as criteria of their biological age. This is a generally useful criterion, but, as Calvert (1929) has pointed out, not wholly valid, because of differences in the numbers, relative lengths, and variation in growth of the instars. Such variations have been recorded as being caused by various environmental factors such as starvation, temperature, humidity, etc. An investigation of *Melanoplus differentialis* from the point of view of its molting will show that in the development of this animal, too, the larval instars vary.

1. *Number of instars.*—Uvarov (1928*b*) reports that "most *Acrididae* have five larval stages but this is not a universal rule." He then cites numerous exceptions in various genera of the family, such as *Dociostaurus kraussi*, a species wherein the male shows four larval stages and the female five. *Hieroglyphus*

banian exhibits six instars in the case of the males, and seven in the case of the females. *Colemania sphenaroides* is mentioned as a species which may go through five or six stages irrespective of sex. Similarly Carothers (1923) mentions three out of seven *Hespe-*

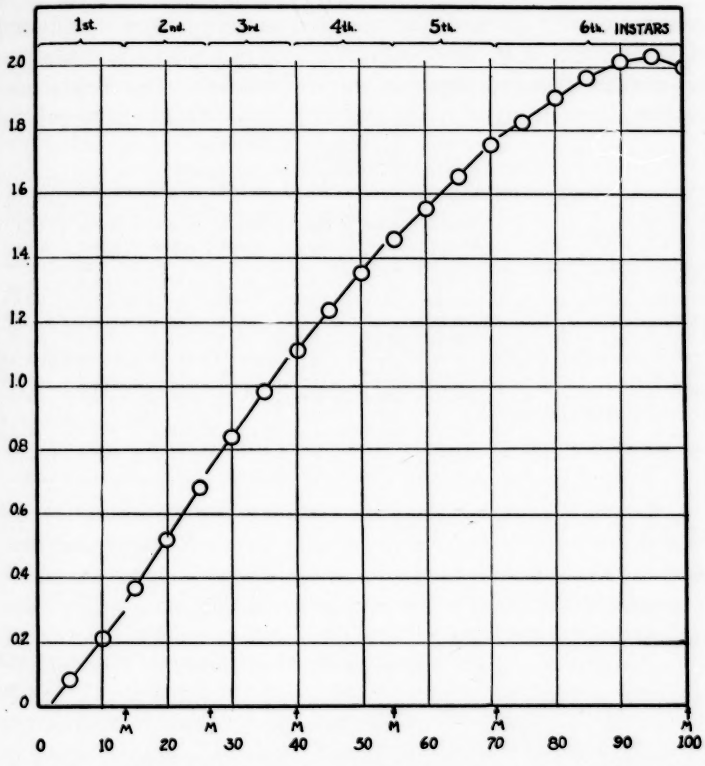

FIG. 8.—Average Growth Rate Curve. Abscissae as in Figure 1. Ordinates—logarithm of the percentage of the transforming weight. Average duration of instars indicated above; average time of molting, below (*M*).

rotettix hybrids (*H. viridis* ×*H. pratensis*) which showed an extra instar each in addition to the five characteristic of the group.

The usual number of instars in the case of *Melanoplus differentialis* is six; but 13 of the insects used in these experiments exhibited

an extra instar apiece, making their total seven instead of six, and 2 males molted eight times each. Of these animals exhibiting extra instars, 6 were males; 2 on the mixed diet, 2 on rye, 1 on barley, and 1 on wheat. The other 7 were females; 2 on the mixed diet, 1 on barley, 1 on lettuce, and 3 on wheat. These animals were all under the same conditions of temperature and humidity. This would seem to indicate that in *M. differentialis* the instars are not altogether perfect criteria of biologic age even under the same environmental conditions.

TABLE II

AVERAGE LENGTH OF INSTARS (IN DAYS)

Diet	First Instar	Second Instar	Third Instar	Fourth Instar	Fifth Instar	Extra Instar	Last Instar
Mixed	8.9	8.4	8.0	10.3	11.2	10.7	19.7
Wheat	11.9	8.6	9.1	9.9	11.8	13.3	17.0
Barley	10.8	8.3	11.1	11.1	11.9	12.9	14.3
Rye	10.6	10.1	9.8	12.1	16.4	14.6	21.3
Average satisfactory diets	10.5	8.8	9.5	10.8	12.8	12.9	18.1
Lettuce	10.7	10.7	8.9	14.8	14.7	16.5	23.0
Oat	18.7	9.4	11.8	18.0	21.7	.....	10.0
Average, unsatisfactory diets	14.7	10.0	10.3	16.4	18.2	16.5	16.5
Average, all diets	11.9	9.1	9.8	12.7	14.6	13.6	17.5

2. *Length of instars.*—The time necessary for an insect to pass through one instar is also widely variable. It is well known that temperature, food, and other environmental factors influence the length of instars. But it can also be demonstrated that even on the same foods and under the same cultural conditions there is wide variation in the lengths of the successive instars in *Melanoplus differentialis*, as shown in Table II.

Thus the average length of the instar for *Melanoplus differentialis* is 12.9 days at the temperature used in these experiments. Individual insects, however, vary widely about this average. The difference between the various averages, either for each diet or for each instar, are hardly significant in view of the wide variation in almost every group of figures. One might say that the later instars tend to be the longest.

3. *Increase in weight in each instar.*—It can be demonstrated that, on the average, *Melanoplus differentialis* rather more than doubles its

bulk in each instar. This can readily be shown by comparing the weight on the first day of any instar with that on the last, in any instar except the last. In the case of the last instar, the maximum weight is not the final weight, since considerable excess weight is lost at transformation. In the case of the last instar, the consideration of two sets of figures is of interest—the weight at transformation, and the maximum weight achieved in the instar (the peak of excess weight strikingly shown on the weight-growth curves). If the latter is taken as the final weight for that instar, it is true in the last instar

TABLE III

AVERAGE PERCENTAGE INCREASE IN WEIGHT PER INSTAR

Diet	Sex	First Instar	Second Instar	Third Instar	Fourth Instar	Fifth Instar	Extra Instar	Last Instar*	Last Instar†
Mixed	♂	61.7	182.2	149.2	98.6	119.3	76.3	121.3	77.9
Mixed	♀	92.2	223.4	124.7	113.5	261.4	.....	112.0	79.9
Wheat	♂	125.7	122.1	114.7	124.6	121.2	105.9	97.8	82.9
Wheat	♀	128.2	194.3	107.1	146.3	137.7	112.8	125.6	87.8
Barley	♂	110.6	136.4	40.4	150.6	97.8	130.2	82.8	63.1
Barley	♀	82.4	180.5	70.8	86.0	147.7	95.3	125.6	107.0
Rye	♂	150.7	100.9	167.9	89.5	93.1	.....	100.5	67.1
Rye	?	41.0	305.0	166.3	76.2	295.0	.....	.....	40.8
Lettuce	♂	103.3	122.8	91.3	133.9	155.1	112.7	.....	73.1
Lettuce	♀	113.3	155.2	88.4	133.8	91.2	133.0	85.5	79.6
Average of	♂♂	116.3	132.8	120.4	119.8	116.2	103.0	106.5	75.4
Average of	♀♀	109.1	207.3	110.2	120.5	158.9	113.2	111.5	84.8
Average	both	119.6	162.2	115.7	124.9	135.6	108.9	106.4	78.7

* Percentage increase from beginning of last instar to maximum weight at top of "peak" of pre-transformation weight.

† Percentage increase from beginning of last instar to weight immediately after transformation.

also that the weight rather more than doubles. If the true final weight is considered, the last instar stands out as an exception to the others, in that weight does not double. The increase in weight per instar is shown in Table III, expressed in terms of percentage, obtained by subtracting the initial weight of each instar from the final weight. This difference, divided by the initial weight and multiplied by 100 represents the percentage increase for that instar.

It is noticeable that the amount of increase is extremely variable. It was pointed out by Dyar (1890) that among certain insects the linear dimensions increase in terms of geometric progression; and subsequently Bodenheimer (1927) has verified this fact for still other

species, attempting to adduce such regularity of growth to agreement with Przibram's (1912) theory that growth necessitates the assumption of definite numbers of cell divisions in each instar, leading to ecdysis after a rather definite number of mitoses. Each instar should show then an increase in weight to be expressed fairly accurately by some multiple of two.

The figures for *Melanoplus differentialis* vary rather widely from such values. Examination of Table III shows that in many cases the weight is nearly doubled within an instar, but, in many more, ecdysis occurs after surprisingly little increase in weight, and in other cases the weight is much more than doubled or occasionally tripled.

Such variation has been pointed out among other species by Teissier (1928); and one is led to the conclusion that in *Melanoplus differentialis* as in various of the species used by the last-named writer "the experiments . . . . hardly permit one to follow Przibram in his interpretation. The law which he proposes . . . . has perhaps a certain statistical value but has assuredly no explicative value" (p. 905, *loc. cit.*).

4. *Linear growth in each instar.*—Various writers have measured the linear growth of insects and attempted to correlate the amount of growth in each instar with theoretical considerations. W. K. Brooks, as cited by Calvert (1929), found a rather constant increase in linear size in the larvae of arthropods of another class—the stomatopod Crustacea—which could be expressed by multiplying the length in any stage by 1.25 in order to obtain the length of the succeeding stage. Przibram and Megusar (1912) determined the corresponding factor for the expression of the linear growth of *Sphodromantis bioculata* Burm. to be 1.26, and found that the weight in each instar is doubled. These writers were led to conclude that the basic cause of the doubling in weight is a doubling in number of cells by mitosis. Each instar showing such a doubling of weight and linear increase by this factor 1.26 (the cube root of 2) they term a true "division step" or *Teilungschritt*.

Such a doubling in weight—or linear increase by 1.26—does not invariably occur in the development of all insects, by any means. Alpatov (1929) finds that *Drosophila melanogaster* shows "three sharply defined instars characterized by the structure of the mouth

parts and respiratory organs (anterior spiracles)," but finds that the relations between the linear dimensions of the mouth parts of two successive stages can be better expressed by the coefficient $(\sqrt[3]{2})^2$ than by $\sqrt[3]{2}$. Accordingly he concludes "that each of the two larval moultings is accompanied by two divisions of the body cells." The same idea is used by Bodenheimer (1927) in his analysis of the linear growth of various insects, from different orders of the class, wherein he is led to the conclusion "that in primitive paurometabolous insects each moult represents a division step. In the *Acrididae* there is a tendency to latent division steps without intervening moults. In *Rhynchota* this is still more common; while in the holometabolous insects with soft-skinned larvae it is very common." According to this theory an indication of the primitive number of instars in the phylogeny of a derived species or group might be had from the increase in size per instar and consideration of latent division steps.

In this study of *Melanoplus differentialis* 143 exuviae from 37 different animals were preserved in 50 per cent alcohol and the length of the left third femur of each measured as accurately as possible in alcohol under a binocular dissecting microscope. By dividing the femur length at each molt into the corresponding dimension of the next exuvia, a factor for linear growth was obtained which has the average value of 1.33 for all the exuviae regardless of sex, diet, or instar in question. Variation between the individual exuviae was considerable. The lowest value obtained was 1.08; the highest, 1.64. No significant difference was shown by comparing the examples on different diets or in different instars. The average values of these factors so derived are shown in Table IV.

In view of the small number of individuals measured, it is believed that this factor for linear growth, 1.33, thus obtained, does not differ significantly from the theoretically expected $\sqrt[3]{2}$. Certainly it is nearer $\sqrt[3]{2}$ than the $(\sqrt[3]{2})^2$ found by Alpatov (1929), Bodenheimer (1927), or others for the animals believed to develop by "doubled" instars or latent division steps. According to the division step or "Teilungschritt" theory, *Melanoplus differentialis* would be interpreted as an insect which develops regularly by six instars, each one of which represents a division step.

But the wide variation in these factors and the irregularity of oc-

currence of the variations lead one, perhaps, to question the validity of assuming that the fundamental cause of ecdysis is to be so easily explained. Growth and development are complex phenomena, and individual variation is considerable. Such growth factors should be considered as convenient measures of development "of statistical value but . . . . assuredly no explicative value" (Teissier, 1928, *loc. cit.*).

TABLE IV

FACTORS EXPRESSING AVERAGE LINEAR GROWTH PER INSTAR

Diet	First Instar	Second Instar	Third Instar	Fourth Instar	Fifth Instar	Sixth Instar	Average
Wheat	*	1.46	1.35	1.32	1.23	1.18	1.31
Barley	*	1.30	1.37	1.31	1.34	1.35	1.33
Rye	*	1.22	1.43	1.34	1.29	1.27	1.31
Lettuce	*	1.31	1.39	1.31	1.33	1.34	1.33

* (NOTE.—No factor derived for first instar, since initial measurement would have had to have been made on live nymph, with attendant danger of injury.)

SUMMARY

This preliminary study has indicated certain satisfactory and unsatisfactory diets for *Melanoplus differentialis* Thomas. The relatively poorer development, irregular growth, susceptibility to pathological conditions, and higher mortality of the insect on such restricted diets as lettuce or oat leaves alone indicate that such criteria can be used as indications of dietary deficiency in further experiments with various insufficient diets. The relatively better development of the insect, as shown by general vigor, longevity, and fertility, on the diets of wheat, barley, or rye leaves alone, shows that such single plant diets are clearly sufficient and satisfactory diets for this species.

This investigation has also afforded growth curves for the insect on four different satisfactory diets. The growth data plotted into a single smoothed curve for the entire pre-adult life show a curve beginning with the initial part of a typical S-shaped growth curve, deviating from this by a loss of weight at each molt, particularly the last. Closer examination of these data indicates a sigmoid curve for each instar, as has been recorded for other species by other writers.

An average rate of growth specific for each instar except the last is suggested. It is further pointed out that the last instar shows two or sometimes three rates, the last a decreasing one.

Six larval instars are typical for both sexes of this species, with an occasional individual exhibiting an extra instar interpolated between the fifth and the last. This extra instar may be shown by insects of either sex on any diet.

The instars of this species at the temperatures used average twelve days in duration. There is considerable individual variation. The later instars tend to be longer than the first three or four.

The weight of the insects approximately doubles in each instar, but great variation is shown in this. The variation apparently is not correlated with any particular diet.

In linear dimensions the animals increase on the average 1.33 times over the corresponding linear dimension of the preceding instar. This factor also shows wide variation.

These growth data should prove useful measures of the performance of the insect on various diets to be used in the future, whereby it is hoped that an exact understanding of the nutritional requirements of this species may ultimately be obtained. Such an understanding should be of significance as well for comparative studies of nutrition as for general nutritional theories.

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L'ONTOGENESI DEGLI IBRIDI FRA BUFO VULGARIS E BUFO VIRIDIS

(Con 36 figure e 3 tavole)

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I. INTRODUZIONE

L'IBRIDAZIONE fra le due specie di rospi comuni in Italia, *Bufo vulgaris* Laur. e *B. viridis* Laur. dà, com'è noto, risultati molto diversi a seconda che si usano le uova dell'una o dell'altra specie. In un caso (*B. viridis* ♀ × *B. vulgaris* ♂) si ottengono larve anormali, che muoiono tutte senza raggiungere il periodo della metamorfosi. L'incrocio reciproco, invece (*B. vulgaris* ♀ × *B. viridis* ♂) dà origine a larve che differiscono poco dai controlli e che si rivelano vitali e capaci di superare la metamorfosi.

Il presente lavoro considera le malformazioni che si ottengono negli embrioni del primo incrocio e alcuni risultati riferentisi all'ontogenesi di quelli del secondo. Per amor di brevità, gli ibridi fra *B. viridis* ♀ × *B. vulgaris* ♂ saranno designati come ibridi del primo incrocio, quelli dell'ibridazione reciproca come ibridi del secondo incrocio.

A. L'IBRIDAZIONE ETEROSPECIFICA NEGLI ANURI

Fin dalle ricerche del Pflüger (1882, 1883) e del Born (1883, 1886) è noto come l'ibridazione fra le diverse specie di Anuri dia risultati molto varî e come, generalmente, gli incroci reciproci abbiano successo assai diverso. Parecchie ricerche furono eseguite in seguito per spiegare il diverso comportamento delle varie combinazioni, ed è soprattutto a G. Hertwig (1918) che spetta il merito di avere ben chiarito la situazione. Le sue ricerche, condotte su diversi incroci fra varie specie di Anuri, furono basate soprattutto su misure del volume dei nuclei degli ibridi, e accompagnate da esperimenti di irradiazione degli spermî, e lo condussero a distinguere "ibridi veri" e "ibridi falsi". Nei primi avviene la fusione della cromatina paterna con la materna, nei secondi la cromatina paterna viene invece eliminata e le larve risultanti sono di solito aploidi, o possono acquistare secondariamente il numero diploide di cromosomi, per un meccanismo tuttora ignoto.

Secondo la terminologia proposta dal Hertwig, sarebbero dunque da distinguersi, nell'ibridazione degli Anuri, casi di ibridi veri, o *orthonothi*, che possono dare embrioni vitali o embrioni non vitali (*dysnothi*), e ibridi falsi o *pseudonothi*, cioè embrioni partenogenetici, normalmente aploidi, talvolta diploidi. Con l'irradiazione degli sper-

mî e altri mezzi atti a danneggiare la cromatina, i *dysnothi* possono essere trasformati in *pseudonothi*.

La dimostrazione citologica di questi fatti si deve a una serie di ricerche del Bataillon (1909), di Bataillon e Tchou (1929) e di Tchou (1931). Quest'ultimo, con lo studio di undici combinazioni fra i gameti di specie diverse di Anuri, potè distinguere tutti i gradi fra: (1) eliminazione precoce e completa del pronucleo maschile; (2) partecipazione della cromatina maschile alle prime figure mitotiche e sua conseguente eliminazione, completa o parziale, a stadî diversi; (3) anfimissi iniziale e definitiva, che può essere totale o parziale.

Con questo lavoro i fenomeni citologici che avvengono nelle ibridazioni degli Anuri possono dirsi esaurientemente descritti, e anche qui si possono riconoscere i diversi casi che si incontrano negli Echinodermi, così riassunti da Lillie e Just (1924): (1) mancata reazione dell'uovo allo sperma eterogeneo; (2) reazione corticale senza penetrazione dello spermio; (3) penetrazione senza anfimissi; (4) anfimissi seguita da eliminazione più o meno precoce della cromatina paterna; (5) anfimissi non seguita da eliminazione, ma incompatibilità delle due cromatine, che si manifesta con anomalie dello sviluppo; (6) anfimissi seguita da sviluppo normale e dalla formazione di larve vitali.

Numerose anomalie dello sviluppo si manifestano nei diversi ibridi. Nel caso 3° gli embrioni hanno le caratteristiche degli embrioni partenogenetici aploidi. Nel caso 4° si osservano condizioni estremamente varie, che vanno da casi di sviluppo partenogenetico completo, a casi in cui l'eliminazione della cromatina paterna è tardiva e lo sviluppo è bloccato allo stadio di blastula (stereoblastula) o di gastrula (stereogastrula). Nel caso 5° che, insieme al 6°, più particolarmente ci interessa, si verificano per lo più malformazioni tipiche, analoghe a quelle che si possono ottenere, negli Anfibî e in altri Vertebrati, con altri mezzi sperimentali, ai quali conviene brevemente accennare.

B. LA TERATOGENESI SPERIMENTALE NEI VERTEBRATI

All'azione di particolari stimoli, di natura fisica o chimica, fatti agire, a varî periodi dello sviluppo, su tutto l'embrione, diverse parti o diversi organi di questo rispondono con intensità differente, e ne risultano quelle anomalie che sono conosciute sotto il nome di inibi-

zioni differenziali. Numerosissimi studî sono stati dedicati a questi fatti, e azioni di natura interamente diversa sono state sperimentate su molte specie di Vertebrati e di invertebrati, talchè è possibile oggi una visione abbastanza ampia e completa dei fenomeni, specialmente per quanto riguarda i Vertebrati. Accenneremo ai principali risultati ottenuti, specialmente sugli Anfibî, rimandando, per maggiori particolari ai lavori speciali, in cui l'argomento e la bibliografia sono più ampiamente trattati.

Anomalie che, come vedremo si possono ricondurre tutte ad alcuni tipi principali, sono state ottenute principalmente in tre modi: (1) con mezzi chimici (sali minerali diversi, anestetici, alcaloidi, ecc.); (2) con mezzi fisici (temperatura, gravità, pressione osmotica, irradiazione, ecc.); (3) con l'ibridazione eterospecifica. È interessante il fatto che le azioni fisiche e chimiche possono produrre effetti sia se applicate direttamente sull'uovo fecondato o sull'embrione, sia se applicate sulle cellule germinali prima della fecondazione.

1. *Con azioni chimiche e fisiche.*—Fra i lavori principali riferentisi ad alterazioni ottenute con il trattamento delle uova fecondate o degli embrioni di Anfibî con soluzioni di sali, anestetici, veleni, alcaloidi, prodotti del catabolismo, ecc., ricorderemo quelli di Morgan (1903), O. Hertwig (1895), Gurwitsch (1895–1896), Wilson, Bataillon, (1901, 1904), Rondeau-Luzeau, Jenkinson (1906). Questi descrissero un gran numero di anomalie che si possono riassumere in due categorie: alterazioni della gastrulazione, con formazione di embrioni anulari, spine bifide, emiembrioni laterali, casi di asintassia del blastoporo, ecc.; e inibizione dei processi organogenetici successivi, con mancata chiusura delle pieghe midollari, inibizione dell'encefalo e degli organi di senso cefalici, del cuore e del sistema circolatorio.

Più recentemente il Leplat (1913, 1919) e il Cotronei (1915, 1919, 1921, 1922*a*, *b*, e *c*) analizzarono le malformazioni ottenute negli embrioni di Anfibî sottoposti all'azione di soluzioni di sali di litio. Il Cotronei, che si valse di questo metodo per mettere in evidenza le correlazioni di varia natura che governano lo sviluppo dei diversi organi, descrisse: riduzione dell'encefalo precordale, malformazioni degli occhi, avvicinamento degli abbozzi oculari fino a completa fusione (ciclopia) accompagnati da inibizioni del vestibolo orale, delle fossette nasali, ecc.

Il Bellamy (1919) prese in esame le malformazioni indotte nello sviluppo di *Rana pipiens* da una numerosa serie di sali, acidi, basi e sostanze organiche diverse, indugiandosi soprattutto sui primi stadî di sviluppo. Descrisse un ritardo nella divisione delle cellule del polo animale, tale da produrre micromeri quasi grandi quanto i macromeri, diversi gradi di inibizione della gastrulazione, che culminano con la formazione di gastrule equatoriali. In un altro lavoro (1922) si occupò degli stadî successivi, e ricordó: microcefalia, occhi, fossette olfattorie e organi adesivi in diverso grado di avvicinamento fino alla completa fusione, forme anencefale e diversi gradi di spina bifida, inibizioni di vario grado nelle regioni del tronco e della coda. Tutte queste alterazioni tentò di spiegare secondo i principî della teoria del Child, a cui pure si riferí il Cotronei (1922*c*).

Anomalie che rientrano essenzialmente nello stesso quadro, ottenute nei Teleostei, con metodi analoghi, hanno fatto oggetto di una serie di ricerche dello Stockard (1906–21) e di altri (McClendon, J. Loeb, Reagan, Gee, Werber, ecc.). Malformazioni simili furono pure ottenute con i sali di litio, dal Ranzi (1929*a*) nei Ciclostomi. Anche nel pollo, Dareste, Férét, Ferret, ecc. ottennero, con azioni analoghe a quelle su accennate, malformazioni dello stesso tipo, e nei Mammiferi, con intossicazione alcoolica acuta dei genitori Stockard e Craigh (1904) provocarono la formazione di mostruosità nella regione cefalica.

Anche con l'azione di certe sostanze sulle cellule germinali prima della fecondazione si ottengono risultati simili (G. e P. Hertwig, 1913; Gee, 1916, G. Hertwig, 1924).

Fra gli stimoli di natura fisica uno dei più usati, per la nettezza dei risultati e la possibilità di agire con dosi determinate, è stato la irradiazione con raggi X o U.V. Bohn, Schaper (1904), Levy, Bardeen (1909) esposero gli embrioni o i gameti prima della fecondazione all'azione del radio e ottennero anomalie simili a quelle che furono poi ampiamente descritte nella classica monografia sulla *Radiumkrankheit* di O. Hertwig (1911). Questo, irradiando le uova fecondate o gli spermî di *Rana fusca*, e G. Hertwig (1911) irradiando le uova prima della fecondazione, ottennero, con dosi e esposizioni diverse, una completa serie di malformazioni, che vanno da disturbi della gastrulazione (embrioni anulari, spina bifida, asintassia del blastoporo) alla formazione di embrioni anencefali, o che mostrano diversi gradi di

inibizione dell'encefalo e degli organi di senso, del cuore e del sistema circolatorio, e delle regioni dorsale e caudale. O. Hertwig descrisse poi alterazioni istologiche soprattutto evidenti nelle cellule nervose.

In seguito il Baldwin descrisse casi di spina bifida e embrioni contorti ottenuti con l'esposizione dell'uovo fecondato di Anfibî ai raggi U.V. e Pasquini e Meldolesi (1929, 1930) determinarono i periodi di massima suscettibilità delle uova di rana durante le varie fasi dello sviluppo all'azione della radioemanazione ("radon"). Pasquini (1931*a* e *b*, 1932) distinse fra le alterazioni della istogenesi e quelle della morfogenesi: le prime dipendono essenzialmente dalla dose di irradiazione, le seconde dallo stadio irradiato. Le modificazioni della istogenesi possono, con dosi opportune, verificarsi anche senza che appaiano notevoli disturbi della organogenesi.

Le ricerche di Oppermann sulla trota con irradiazione degli spermî, della Hinrichs (1925) con i raggi U.V. su *Fundulus*, del Ranzi su *Petromyzon*, diedero risultati analoghi a quelli ottenuti sugli Anfibî e così dicasi dei lavori di Tur con il radio, di Gilman e Batjer con i raggi X e della Hinrichs (1927) con i raggi U.V. su embrioni di pollo.

Anche l'azione della gravità, la cui direzione può essere modificata con il capovolgimento e l'intensità con la centrifugazione, ha sensibilissimi effetti sullo sviluppo embrionale. In una recente analisi degli effetti della centrifugazione sui primi stadî embrionali di rana, Pasquini e Reverberi (1929) mettono in rilievo, fra l'altro, il fatto che le anomalie ottenute non sono specifiche della centrifugazione, ma presentano moltissimi punti di contatto con quelle che si ottengono con agenti inibitori di altra natura.

Lo stesso si può dire per le condizioni di temperatura fatte agire in tempi e per durate opportune sia sull'uovo degli Anfibî (Jenkinson, 1909) che su quello di Pesci (Loeb, 1915; Kellicott) e di Uccelli (Alsop).

2. *In seguito a ibridazione.*—Le conoscenze più precise sulla produzione di mostri con la ibridazione si riferiscono ai Teleostei, e in particolare al genere *Fundulus*. Il Moenkhaus (1910) descrisse alcuni teratomi che si ottengono con l'ibridazione fra *Fundulus* e *Menidia*, che furono poi ripresi in esame dal Loeb (1915). Casi simili sono ricordati anche, per altre specie, da G. e P. Hertwig (1914); ma lo studio più completo si deve al Newman (1908–1917) che analizzò

i risultati della ibridazione fra numerose specie di pesci. Nel lavoro riassuntivo sull'argomento della teratogenesi (Newman, 1917) egli ricorda come le mostruosità ottenute interessino specialmente il sistema nervoso, gli organi di senso cefalici e il cuore. In accordo con i principî e la terminologia del Child, le classifica in: (*a*) forme risultanti da inibizione differenziale; (*b*) forme risultanti da acclimazione o ristabilimento differenziale. Come casi estremi di quest'ultima categoria ricorda occhi o cuori isolati, cresciuti su di un blastoderma indifferenziato, embrioni con la parte cefalica fortemente inibita e la caudale ben sviluppata, o viceversa.

La somiglianza di questi mostri con quelli ottenuti con altri mezzi sperimentali indusse il Newman a contraddire l'opinione, accettata da varî autori e ricomparsa anche in lavori più recenti, di una specificità di certi agenti nel produrre particolari teratomi. Riconobbe così che la stessa serie di teratomi può essere prodotta in tre modi: "1. By use of chemicals. . . . . 2. By means of low temperature. . . . . 3. By means of heterogenic hybridisation. . . . ."

Negli ibridi di pesci, anche quando le uova di una sola femmina vengano fecondate da spermî di un solo maschio, si può ottenere un'ampia serie di mostruosità di grado molto diverso, e, in certi casi, embrioni mostruosi, embrioni simili ai normali, e embrioni meglio sviluppati e, a parità di condizioni, più precoci, grossi e resistenti dei normali. L'autore potè stabilire una relazione fra la velocità di sviluppo e questi varî casi: a seconda che lo sviluppo è più o meno rallentato, gli embrioni risultano più o meno fortemente inibiti, se è accelerato, gli embrioni sono anche meglio sviluppati del normale. Il Newman giunse così alle seguenti conclusioni: "there is a very close relation between the rate of development and the degree of normality of the embryo" e "in introducing a foreign sperm into an egg we either accelerate or retard the developmental rate of the egg." Ritiene quindi che l'azione comune a tutti gli agenti teratogeni sia un rallentamento della velocità di sviluppo. Alle stesse conclusioni giunse più tardi lo Stockard, in base agli studî su ricordati, e dopo avere abbandonato l'idea della specificità, che aveva sostenuto in lavori precedenti. Nel suo lavoro del 1921, infatti, così si esprime: "the primary action of all the treatments is to inhibit the rate of development."

In successive ricerche sugli ibridi fra *Strongylocentrotus purpuratus* e *S. franciscanus*, il Newman (1923) confermò ed estese i suoi risultati anche agli Echinodermi.

I lavori principali sugli Anfibî sono quelli già citati del Hertwig e del Tchou. Questi però non si indugiano a descrivere i risultati dell'azione inibitrice che si manifesta in alcuni incroci, e si limitano ad accennare di sfuggita ad alcuni dei casi ottenuti. G. Hertwig ricorda, brevemente, per l'incrocio *B. viridis* ♀ × *B. vulgaris* ♂, casi di spina bifida, asintassia del blastoporo, embrioni nani, con notevole perdita di tuorlo e deformati in modo caratteristico, di cui nessuno potè superare i 12 giorni d'età.

Da questa sommaria esposizione di alcuni principali lavori sulla teratogenesi sperimentale dei Vertebrati si possono trarre alcune considerazioni generali, che conviene tener presenti per il nostro scopo.

L'azione teratogena di stimoli di natura assai diversa dà risultati essenzialmente analoghi: si ammette generalmente che a disturbi più gravi corrispondano anomalie della gastrulazione, che possono manifestarsi in modo alquanto diverso a seconda della intensità dell'azione, non solo, ma del tipo di segmentazione e di sviluppo. A più lievi disturbi seguono invece malformazioni localizzate principalmente nella regione cefalica e particolarmente nel sistema nervoso, nonchè nel cuore e nel sistema circolatorio. Le varie anomalie che si ottengono sono perciò da considerarsi quantitativamente piuttosto che qualitativamente differenti. Le numerose ricerche eseguite sui Ciclostomi, Teleostei, Anfibî, Uccelli, e alcuni dati relativi ai Mammiferi, permettono di generalizzare questa conclusione a tutti i Vertebrati, qualora si tenga conto delle particolarità di sviluppo nei singoli casi. Deve quindi essere abbandonata l'idea della specificità dei singoli reattivi come determinanti certi tipi di malformazioni, almeno nella forma in cui era ammessa dai primi sperimentatori. La specificità è piuttosto nel modo di reazione dell'organismo, essenzialmente simile verso stimoli diversi, e uguale, nelle linee generali, in tutta la serie dei Vertebrati. Azione comune a tutti i mezzi finora sperimentati sembra essere un rallentamento dello sviluppo.

In un certo senso, tuttavia, non si può del tutto escludere la spe-

cificità del reattivo per certi tipi di anomalie. Il cloruro di magnesio, ad es. si è dimostrato un ottimo produttore della ciclopia in *Fundulus*, mentre con altri sali si inducono più facilmente inibizioni più diffuse. Con l'irradiazione, generalmente, non si ottiene la ciclopia negli Anfibî mentre è facile determinarla con il cloruro di litio, ecc. Quindi, se, in generale, si può dire che l'azione dei varî agenti teratogeni dà risultati simili, non si può affermare che le malformazioni risultanti, sullo stesso materiale, siano identiche, e conviene ammettere una certa elettività nell'azione esercitata. È opportuno osservare, a questo proposito, che, mentre con alcuni reattivi, specialmente con i sali, si ottengono anomalie estremamente localizzate (es. ciclopia) e il resto del corpo può rimanere quasi normale, con azioni di altro genere (es. irradiazione) le anomalie appaiono più diffuse e interessano, più o meno gravemente, tutte le regioni. Il Pasquini (1931, *a*, *b*, 1932) mise in rilievo come con la radioemanazione sia possibile differenziare l'azione specifica (*Radiumkrankheit*) da quelle non specifiche (malformazioni della organogenesi).

La gravità delle anomalie, in genere, cresce con l'aumentare della dose del reattivo; con dosi medie si ottengono però serie abbastanza ampie di inibizioni. Ciò si può verificare soprattutto con esperimenti condotti su stadî rigorosamente paragonabili, e, meglio, con azioni dirette sui gameti, prima della fecondazione, perchè la suscettibilità degli embrioni e dei singoli organi varia considerevolmente nei diversi stadî di sviluppo e i tipi di malformazione che si ottengono sono perciò dipendenti anche dal momento in cui avviene l'intervento sperimentale.

II. MATERIALE E METODO. TABELLA DEGLI ESPERIMENTI

Diverse serie di esperimenti furono eseguite durante la primavera del 1929 e quella del 1930. Il materiale che ha fornito oggetto per questo studio è principalmente quello esposto nella tabella I.

Gli animali provenivano dalla campagna romana dove ambedue le specie sono comunissime. Catturati poco prima del tempo della riproduzione, si accoppiano facilmente e depongono le uova in cattività.

Non appena la femmina cominciava a deporre le uova, veniva separata dal maschio, uccisa, e l'epidermide accuratamente disinfet-

tata. Incisa e aperta la cavità addominale, le uova, già disposte nel cordone gelatinoso, venivano estratte dall'utero con strumenti sterilizzati, e tratti del cordone comprendenti all'incirca un centinaio di uova erano posti in scatole di Petri pure sterili. Gli spermî erano invece ottenuti per dilacerazione dei testicoli in acqua di fonte; la sospensione lattea, opportunamente diluita, veniva poi spruzzata sulle uova per mezzo di pipette. I controlli di uova normalmente fe-

TABELLA I

PROSPETTO DEGLI ESPERIMENTI ESEGUITI

Incrocio	Data della Fecondazione	Data della Fissazione	Designazione	Età
Bufo viridis ♀ ×*B. vulgaris* ♂	12. IV. 1930	17. IV	*Buv. A*	5 giorni
		24. IV	*Buv. B*	12 giorni
	13. IV. 1930	17. IV	*Buv. C*	4 giorni
		30. IV	*Buv. D*	17 giorni
		1. V	*Buv. E*	18 giorni
		4. V	*Buv. F*	22 giorni
	3. VI. 1930	4. VI	*Buv. G, H*	1 giorno
		5. VI	*Buv. I, J, K, L*	2 giorni
		7. VI	*Buv. M*	4 giorni
		10. VI	*Buv. N*	7 giorni
		21. VI	*Buv. O, P*	18 giorni
B. vulgaris ♀ ×*B. viridis* ♂	13. IV. 1930	17. IV	*Buc. Q*	4 giorni
		1. V	*Buc. R*	18 giorni
		20. V	*Buc. S*	37 giorni
		21. V	*Buc. T*	38 giorni
		24. V	*Buc. U*	42 giorni
		12. VI	*Buc. V*	2 mesi

condate furono tenuti sempre nelle stesse condizioni di luce, temperatura, alimentazione a cui erano sottoposti gli ibridi.

Embrioni e girini furono fissati per la maggior parte in sublimato acetico, alcuni in liquido di Zenker, e le sezioni, di 7–8 μ, colorate con ematossilina o emallume e eosina.

Gli esperimenti del 13. IV e quello del 3. VI furono eseguiti con uova di una sola femmina fecondate con spermî di un solo maschio. Gli embrioni su cui è basata la descrizione che segue sono, per il primo incrocio, soprattutto quelli della fecondazione fatta il 3 giugno. A quell'epoca la temperatura era piuttosto elevata e lo sviluppo av-

venne con velocità sensibilmente maggiore che non negli esperimenti precedenti.

I controlli sono indicati con la stessa notazione: *Contr. Buv. A*, *B*. . . . . ecc.

III. CENNI SULLO SVILUPPO NORMALE DELLE DUE SPECIE PARENTI

L'uovo di *Bufo vulgaris* misura due millimetri, o poco più, di diametro, è intensamente pigmentato, con l'area chiara del polo vegetativo assai ridotta. L'uovo di *B. viridis* è più piccolo (1.3–1.5 mm. di diametro) ha pigmentazione meno intensa e l'area chiara del polo vegetativo più ampia. Anche gli spermî del *B. vulgaris* sono un poco più grossi di quelli del *viridis*, come risulta anche dalle microfotografie pubblicate da G. Hertwig (1930).

La velocità di sviluppo delle due specie è diversa: due lotti di uova furono fecondati, ciascuno con spermî della propria specie, alle 12.55′ del 13. IV. 1930. Alle 16.20′ comparve il primo solco nelle uova del *viridis*, e alle 16.30′ tutte erano divise in due blastomeri. Soltanto alle 17 invece comparve il primo solco nelle uova del *vulgaris*. La distanza si mantenne in seguito, nel corso dello sviluppo: gli embrioni del *viridis*, a parità di condizioni esterne erano sempre un poco più avanti nello sviluppo. G. Hertwig ha dimostrato che, nonostante che il volume delle cellule sia minore in *viridis* che in *vulgaris*, il rapporto carioplasmatico è eguale nelle due specie; è perciò ragionevole supporre che lo sia pure nelle uova, e questa differenza nella velocità di segmentazione e dello sviluppo non deve quindi essere semplicemente dovuta alla maggiore quantità di deutoplasma dell'uovo di *vulgaris*, ma corrispondere a differenze fisiologiche caratteristiche delle due specie.

La percentuale delle uova che si orientano, nelle condizioni dell'esperimento è di circa 85–90% in ambedue i casi. Quasi tutte le uova procedono poi regolarmente nello sviluppo, ma quasi sempre alcuni embrioni (1–2%) presentano anomalie diverse, generalmente precoci (spina bifida, gastrulazione anomala) che li conducono a morte. In seguito non si verifica una notevole mortalità, fino all'epoca della metamorfosi, e lo sviluppo procede con notevole regolarità.

I girini del *vulgaris* sono sempre un po' più grossi di quelli del *viridis* di pari età, e la differenza si va poi ancora accentuando in

seguito. Sono più intensamente pigmentati, quasi neri, mentre quelli del *viridis* hanno un colore bruno scuro.

IV. L'INCROCIO BUFO VIRIDIS ♀ × B. VULGARIS ♂

A. SEGMENTAZIONE, GASTRULAZIONE E CHIUSURA DELLE PIEGHE MIDOLLARI

La percentuale delle uova che si orientano non è diversa da quella dei controlli (uova di *viridis* normalmente fecondate). Il primo solco si forma pure contemporaneamente ai controlli, e la segmentazione procede sincrona con questi, a giudicare da quanto si è potuto osservare. Relativamente rari sono i casi di segmentazioni anomale, con prima divisione in tre o più blastomeri, o disturbi che producono blastule con masse indivise di tuorlo.

Si formano così, presso a poco contemporaneamente ai controlli, blastule che hanno, per lo più, apparenza perfettamente normale. Le scarse divisioni anomale portano inevitabilmente alla morte in stadî precedenti la gastrulazione; ma anche alcune delle blastule che non differiscono, apparentemente, dalle normali, si rivelano incapaci di gastrulare, e muoiono, dopo uno o due giorni, senza che sia comparsa la minima traccia del solco dorsale. Sono, probabilmente, stereoblastule, con blastocele assente o ridotto, come quelle che si ottengono con alte dosi di irradiazione e quelle che si incontrano frequentemente in altre ibridazioni di Anfibî.

Con la gastrulazione, il periodo critico della vita embrionale, cominciano a manifestarsi le prime notevoli divergenze fra lo sviluppo dei controlli e quello degli ibridi. In tutti questi, senza eccezione, il processo è ritardato, ma in diversa misura nei varî embrioni. Benchè in alcuni ibridi il solco compaia contemporaneamente ai controlli, il movimento del blastoporo verso il polo vegetativo è più lento e la differenza si rende tosto notevole.

In alcuni embrioni il solco si forma nel piano equatoriale della blastula, si estende tutt'intorno su questo piano, ma non si sposta verso il polo vegetativo. Ne risulta una gastrula equatoriale, con tappo vitellino gigantesco, costituito da quasi mezzo emisfero. L'esame delle sezioni rivela che la invaginazione dei materiali può essere più o meno progredita nei varî casi, e il differenziamento degli organi assili può procedere alquanto, conducendo alla formazione di embrioni anulari. Questi si possono considerare come casi estremi di spina bifida e spesso manifestano varî gradi di asimmetria.

In altri embrioni la invaginazione procede più regolarmente e le labbra del

blastoporo si spostano gradualmente verso il polo vegetativo. Come mostrano le figg. 1–5, il processo avviene con diversa velocità nei varî embrioni, talchè si

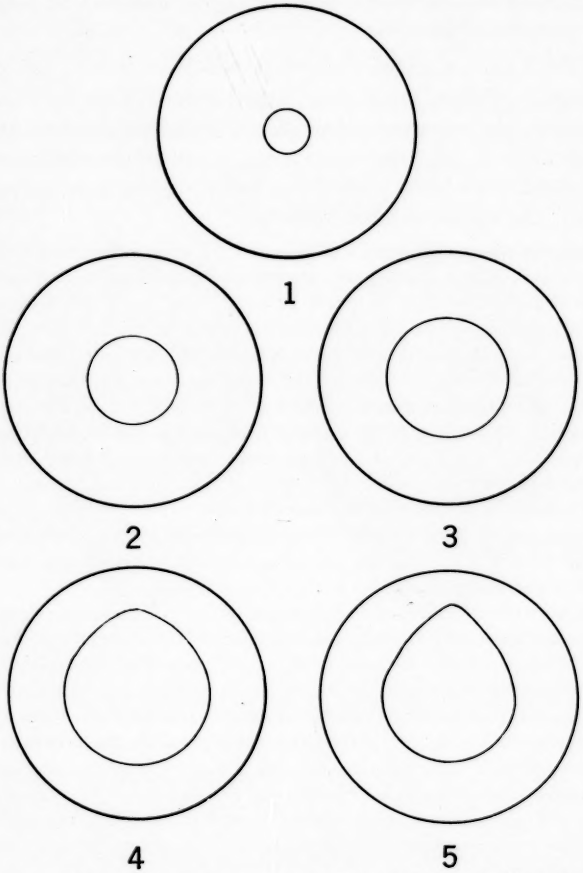


FIG. 1–5.—Schemi di gastrule, provenienti da uova di *Bufo viridis*, viste dal polo vegetativo, che mostrano diversi gradi di estensione del blastoporo; il labbro dorsale è in alto. Fig. 1, controllo (*Contr. Buv. H.*); Fig. 2–5, ibridi (*Buv. H.*) (Tutte le figure, se non è esplicitamente detto il contrario, furono eseguite, col binoculare e la camera lucida, da embrioni fissati.) (Ingr. 20 diam.)

osservano tutti i gradi intermedî fra gastrula equatoriale e gastrula normale. Non di rado si osserva un ritardo maggiore del labbro dorsale del blastoporo, che

assume una forma a V (figg. 4 e 5) come fu descritto dal Bellamy (1919) in uova di *Rana pipiens* trattate con LiCl. Nelle blastule meno inibite il blastoporo ha invece la forma regolare, ma è sempre più largo di quello che, contemporaneamente, presentano i controlli.

Fin dalla gastrulazione dunque si può riconoscere l'esistenza di inibizioni di diverso grado, che si manifestano con disturbi più o meno gravi: dalla assenza totale di invaginazione alla formazione di exogastrule o di gastrule equatoriali, a rallentato movimento del labbro dorsale del blastoporo, fino a uno svolgersi quasi normale del processo, che appare soltanto ritardato.

Le gastrule equatoriali, come abbiamo detto, portano alla formazione di embrioni anulari. Oltre a questi molte altre gravi anomalie, derivanti dall'inibito processo di gastrulazione, si osservano in seguito negli embrioni. Molto comuni sono i casi di spina bifida più o meno pronunziata, in cui la bipartizione della corda e del tubo midollare si inizia più o meno caudalmente all'encefalo. Nei casi più gravi si formano exogastrule, o quelli che il Roux ha chiamato emiembrioni anteriori, con una enorme massa vitellina sporgente dalla parte dorsale. In casi meno gravi invece il tappo vitellino sporge dalla regione caudale e la biforcazione degli organi assili si inizia molto più indietro. Il tipo di spina bifida che di gran lunga prevale è appunto quello in cui il blastoporo ha posizione caudale. Di rado si trova il tipo a blastoporo dorsale, con riunione dei due neurassi e formazione del bottone codale dietro a questo. In certi embrioni più avanzati si nota tuttavia una tendenza alla bifidità del tubo midollare limitata alla regione del tronco, che può essere ricondotta a questo tipo di anomalia.

Tutte queste malformazioni, che si presentano in una vasta serie degradante, sono probabilmente derivate dalle gastrule in cui il labbro dorsale del blastoporo appare ritardato nel suo movimento. Frequenti sono i casi in cui i movimenti della gastrulazione procedono regolarmente, ma tuttavia la chiusura del blastoporo non si completa e il tappo vitellino rimane ampiamente sporgente (asintassia del blastoporo; cfr. fig. 12). Talora l'embrione presenta una lieve biforcazione delle strutture assili nella parte caudale, ma non di rado queste sono normali e può formarsi il bottone codale. L'asintassia del blastoporo può dunque essere correlata alla formazione di spina bifida, oppure il differenziamento degli organi assili e il successivo sviluppo delle forme del corpo può non risentire l'influenza di questo difetto.

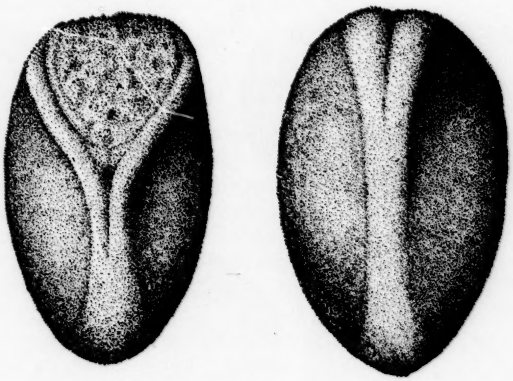
Diversi gradi di asimmetria si manifestano spesso nelle formazioni di questo genere; i più estremi costituiscono emiembrioni laterali, in cui una delle metà del corpo fa interamente difetto ed è sostituita da un'ampia massa vitellina.

Anche quando la gastrulazione si è normalmente compiuta, notevoli anormalità possono insorgere nella chiusura delle pieghe midollari. In taluni embrioni queste rimangono totalmente aperte, in altri la chiusura non procede fino a tutta la regione cefalica, ma si arresta a varî livelli del corpo. Le figg. 6 e 7

mostrano due casi, diversamente gravi, di incompleta chiusura delle pieghe midollari, fra i quali è facile trovare tutti i gradi intermedî. Risultano così, a seconda del livello a cui si arresta la fusione delle pieghe, embrioni anidiani (mancanza totale di chiusura; figg. 11 e 17) embrioni con acefalogenesi completa, o embrioni emicranici, in cui si forma soltanto la terza o la seconda e la terza vescicola cerebrale.

In altri embrioni invece la chiusura delle pieghe neurali si completa più o meno agevolmente, talchè infine questi non differiscono dai normali se non per l'arretrato sviluppo.

Dopo due giorni dalla fecondazione, mentre i controlli erano allo stadio rappresentato dalla fig. 8, con capo e bottone codale bene accennati, le colture degli



6 7

FIG. 6–7.—Due casi di incompleta chiusura delle pieghe midollari (*Buv. I*). (Ingr. 20 diam.)

ibridi offrivano un aspetto assai diverso. Vi si trovavano: exogastrule o emiembrioni anteriori, embrioni anulari, spine bifide di vario grado (fig. 10) ed emiembrioni laterali, casi diversi di asintassia del blastoporo (figg. 10–12), embrioni anidiani o con acefalogenesi più o meno completa, e infine embrioni apparentemente normali, ma più o meno arretrati rispetto ai controlli (fig. 9).

Tutti gli embrioni ibridi emettono, fin dal tempo della gastrulazione, abbondante materiale vitellino, che intorbida lo spazio perivitellino, e, dopo la schiusa (la quale avviene ad uno stadio più precoce che nei controlli) l'acqua del recipiente. Ciò avviene non soltanto negli embrioni che hanno il tappo vitellino sporgente, ma anche in quelli che hanno chiuso il blastoporo, per rottura dell'epidermide ventrale.

Anche nei primi stadî dell'embriogenesi si incontrano dunque, come già nella gastrulazione, diversi gradi di inibizione, che si palesano con la mancanza totale o parziale di chiusura delle pieghe midol-

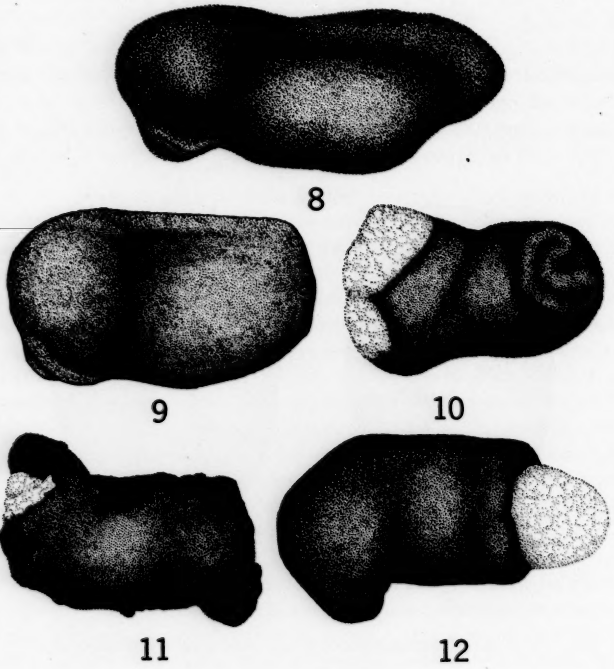


FIG. 8.—Controllo di due giorni (*Contr. Buv. J.*).

FIG. 9.—Embrione *J 4*, d'aspetto quasi normale, ma meno sviluppato del controllo.

FIG. 10.—Embrione *J 2*, con spina bifida, visto dal ventre; cfr. con fig. 23, per gli organi adesivi.

FIG. 11.—Embrione anidiano *M 6;* per il controllo v. fig. 13 (cfr. tav. I, fig. 1).

FIG. 12.—Embrione *J 3*, con forte inibizione della regione cefalica, asintassia del blastoporo, ma organi assili non biforcati.

(Ingr. 19 diam.)

lari, e lo sviluppo più o meno ritardato degli embrioni che hanno condotto a termine questo processo.

B. L'ORGANOGENESI

1. *Forma esterna, organi adesivi, bocca, fossette nasali, branchie.*

Forma esterna: Negli embrioni in cui l'inibizione della gastrulazione ha prodotto anomalie più gravi, come embrioni anulari, spina bifida, emiembrioni anteriori o laterali, l'organogenesi e lo sviluppo delle forme esterne non possono, evidentemente, procedere molto oltre. Gli embrioni anulari non vanno oltre la formazione dell'abbozzo della corda e del tubo midollare. Le spine bifide possono differenziarsi un poco più, a seconda della gravità dell'alterazione. Alcuni casi di emiembrioni laterali possono sviluppare un vestigio di bottone codale, ma si capisce che in tutte queste anomalie l'embriogenesi deve arrestarsi in stadî precoci e gli embrioni muoiono in breve tempo.

Gli embrioni che hanno gastrulato in modo normale e quelli in cui il blastoporo non si è interamente chiuso, senza che la formazione degli organi assili sia stata perciò compromessa, si sviluppano ulteriormente, assumendo ben tosto una forma caratteristica, a "gallinella." Come si vede dalle figg. 14, 15 e 16, le quali rappresentano embrioni di 4 giorni, l'embrione è incurvato dorsalmente sul sacco del tuorlo, talchè la parte di mezzo del dorso forma una sella più o meno profonda. Il capo presenta intensa inibizione della regione rostrale, e gli organi adesivi, generalmente ben sviluppati, sporgono ancor più ventralmente, per la mancata possibilità di sviluppo in senso antero-posteriore. La coda è diretta pressochè verticalmente o obliquamente indietro e l'epidermide del ventre, specialmente nella parte caudale, è fragile e si rompe facilmente, lasciando uscire masserelle di tuorlo. Nei casi di asintassia del blastoporo, il tappo vitellino protrude ampiamente e il tuorlo si va disfacendo; ciò non evita tuttavia la rottura dell'epidermide del ventre. La parte dorsale della pinna può essere sviluppata, come un basso orlo a contorni irregolari e frastagliati, o, in alcuni casi, mancare del tutto; la parte ventrale generalmente fa difetto.

Il colore degli embrioni è un brunastro più chiaro che nei controlli, specialmente sul tuorlo, dove diviene gialliccio. L'epidermide presenta increspature e tubercoli in tutte le regioni del corpo.

Oltre a embrioni di questo tipo, se ne trovano contemporaneamente altri meno fortemente inibiti (fig. 18). Sono infatti alquanto più grossi e, benchè molto in arretrato rispetto ai controlli, presentano aspetto più normale: la insellatura dorsale è poco pronunziata o assente e la coda, assai corta, ha una posizione più vicina alla normale.

Tale è l'aspetto delle culture degli ibridi alla fine del quarto giorno. Molti degli embrioni più inibiti muoiono nel corso dei giorni successivi; altri mostrano ancora qualche progresso, trasformandosi in mostri del tipo di quello riprodotto in fig. 21. Alcuni presentano un ulteriore sviluppo soprattutto nella regione caudale e si trasformano in mostri simili a quelli rappresentati nelle figg. 19, 20, 22, nei quali la inibizione della regione rostrale si rivela sempre gravissima. Gli embrioni meno inibiti invece si sviluppano ulteriormente e si trasformano in girini

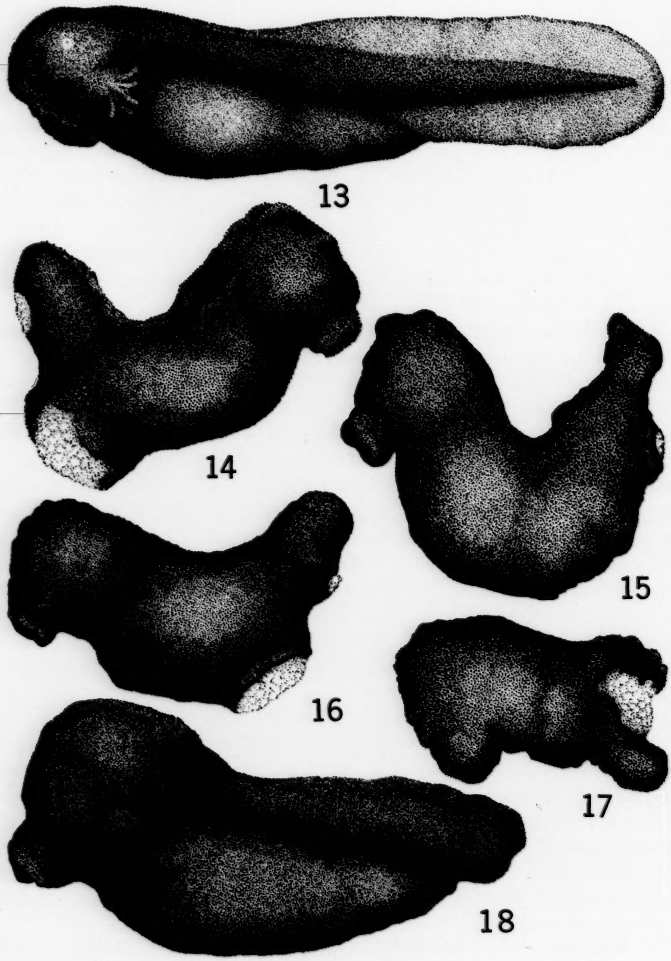


FIG. 13.—Controllo di 4 giorni (*Contr. M*).

FIG. 14.—Embrione *M 7*.

FIG. 15.—Embrione *M 1* (cfr. tav. I, fig. 2).

FIG. 16.—Embrione *M 3*, tutti con forte inibizione della regione cefalica e curvatura dorsale accentuata.

FIG. 17.—Embrione *M 2*, anidiano.

FIG. 18.—Embrione *M 4* (cfr. tav. I, fig. 4, e tav. II, fig. 5), meno gravemente inibito dei precedenti

(Ingr. 19 diam.)

più simili ai normali, con coda meglio sviluppata e inibizione della regione rostrale meno intensa.

Al settimo giorno, oltre a mostri come quelli rappresentati nelle figure citate, si trovano parecchi girini che hanno aspetto quasi normale, benchè siano sempre meno sviluppati dei controlli. Qui ancora si possono riconoscere, all'aspetto della parte apicale del capo (v. oltre) e al profilo del dorso e della coda, varî gradi di malformazione, fino a un tipo che non è esternamente distinguibile dal normale. Questo è rappresentato, peraltro, da un numero estremamente esiguo di individui.

Mentre nessuno dei girini del tipo di quelli rappresentati nelle figg. 19–22 sorpassa i 12–15 giorni di età e tutti muoiono senza che si sia verificato un notevole progresso nello svilluppo, dopo avere non di rado manifestato idropisia, alcuni girini meno inibiti possono vivere fino al 18°, 20°, 25° giorno, o anche più. Ciò si è verificato soprattutto nell'esperimento del 3. VI, dove la percentuale dei girini meglio sviluppati è stata notevolmente superiore a quella ottenuta nelle ibridazioni fatte nei mesi precedenti. Poichè la temperatura era piuttosto elevata e lo sviluppo era avvenuto con velocità molto maggiore che negli esperimenti dell'aprile, si può supporre che la accelerazione determinata dalla temperatura abbia in certo modo compensato i risultati della inibizione, facilitando il migliore sviluppo delle parti inibite.

Anche questi individui meglio formati, benchè capaci di alimentarsi, si mostrano però molto meno resistenti dei normali. Non tardano a manifestare sintomi di idropisia e nessuno si avvicina alla metamorfosi. In nessun caso infatti si è avuto il minimo accenno di formazione degli arti posteriori. È possible che la tiroidina, usata con un certo successo dal Cotronei (1923) per provocare la metamorfosi delle larve inibite con i sali di litio, possa agire favorevolmente anche in questi casi. Soltanto con mezzi di questo genere si può sperare di aver modo di studiare in questi ibridi il comportamento di quei caratteri ereditarî che compaiono dopo la metamorfosi.

È d'uopo rilevare, infine che le dimensioni di tutti i girini sono sempre inferiori a quelle dei controlli e appaiono correlate con il grado di inibizione: quanto più profonde le malformazioni della regione cefalica, tanto più piccoli gli embrioni, a paragone dei loro confratelli coetanei.

Organi adesivi: Gli organi adesivi delle larve di *Bufo* si originano come un solco semilunare nell'epidermide della regione ventrale del capo. In seguito l'epidermide si ispessisce, cranialmente e caudalmente al solco, e questo assume una forma di V (cfr. Marchetti, 1914*a*). La continuità dell'ispessimento caudale si rompe poi, e infine l'organo assume la forma di una M (figg. 23, 25) le cui branche, dapprima accostate, vanno poi man mano allontanandosi l'una dall'altra, nel corso dello sviluppo.

Negli ibridi l'abbozzo di questi organi è sempre visibile, in tutti i casi in cui si ha un differenziamento, sia pure rudimentale, della regione cefalica. Nelle spine bifide, negli emiembrioni e nei casi di più forte inibizione, l'abbozzo degli organi adesivi si presenta generalmente come una piccola protuberanza semilunare, che

può essere notevolmente asimmetrica negli emiembrioni e che, negli individui un po' meglio differenziati, può presentare un solco a forma di V (fig. 10). Le due branche esterne della M rimangono però fuse caudalmente.

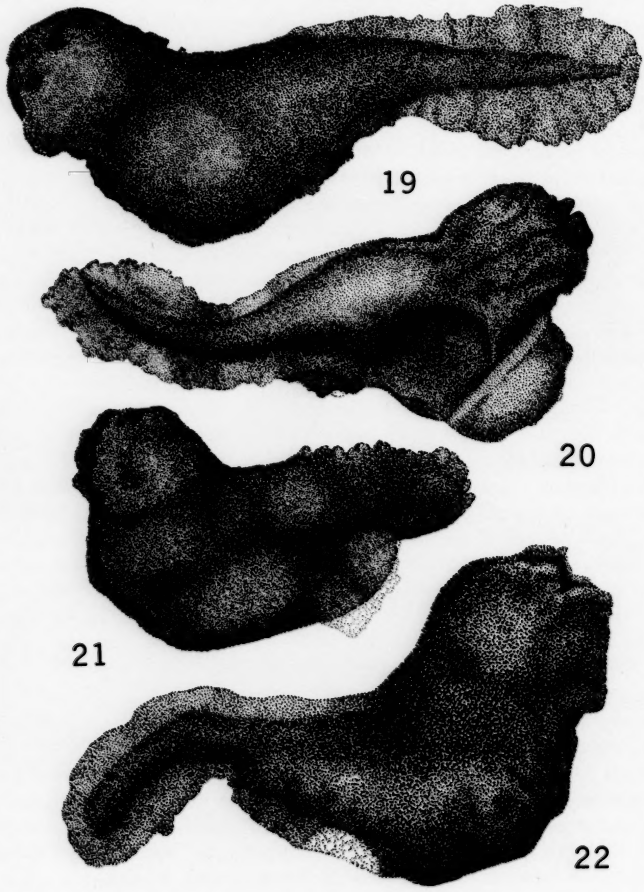


FIG. 19.—Embrione *N 2*, di 7 giorni (cfr. tav. III, fig. 1).
FIG. 20.—Embrione *N 4*, (cfr. tav. III, figg. 10–12).
FIG. 21.—Embrione *N 3*.
FIG. 22.—Embrione *N 1* (cfr. tav. III, figg. 13–14).
(Ingr. 19 diam.).

Negli embrioni meno profondamente alterati, gli organi adesivi non sono dissimili dai normali se non per le dimensioni e per il fatto che appaiono come compressi in senso trasversale, sono ispessiti e sporgono sul profilo ventrale molto più che non negli embrioni normali (fig. 24).

In generale questi organi non appaiono molto ridotti e mostrano una notevole capacità di differenziarsi, acquistando pure la facoltà di secernere muco, anche nei casi di malformazioni più gravi. In quelle larve che più progrediscono nello sviluppo hanno aspetto molto simile a quello dei controlli dello stesso stadio, e si riducono poi, e al loro posto rimane una sorta di cicatrice con cellule più intensamente pigmentate, come nei girini normali.

23 24 25

FIG. 23.—Testa del *Contr. J*, di due giorni (fig. 8), vista dalla parte ventrale.

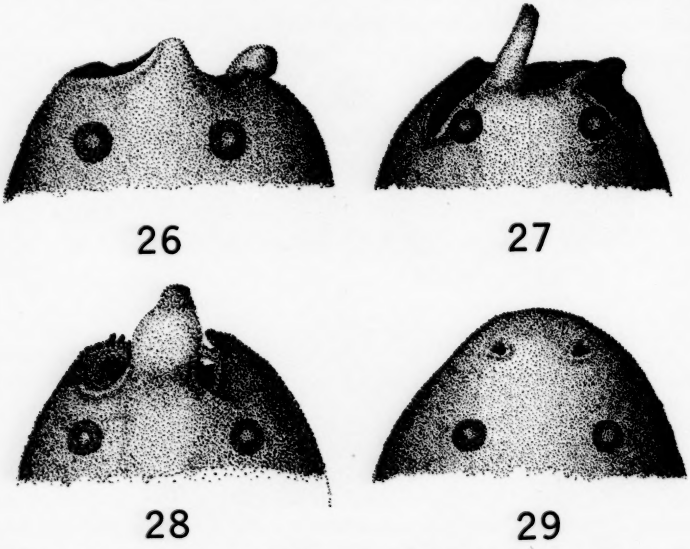
FIG. 24.—Testa dell'embrione *M 6*, di 4 giorni, vista dalla parte ventrale; si vedono gli organi adesivi e il tubercolo impervio, provvisto di papille, che tien luogo della bocca.

FIG. 25.—Testa del *Contr. M* (fig. 13) di 4 giorni, vista dalla parte ventrale. (Ingr. 20 diam.)

Vestibolo orale e fossette nasali: Sono queste le parti più profondamente alterate dalla inibizione, che colpisce particolarmente la regione rostrale. Negli embrioni di 4 giorni, quando già i controlli hanno il vestibolo orale aperto e comunicante con l'intestino, allo stadio rappresentato dalla fig. 25, negli ibridi per lo più non se ne vede traccia. In alcuni un piccolo tubercolo fornito di minuscoli abbozzi di papille rappresenta la bocca, impervia, notevolmente spostata in senso dorsale, come rivela la sua distanza dagli organi adesivi (fig. 24). Altri, come *M 4* e *5*, sono invece in una condizione che ricorda quella dei controlli di due giorni (fig. 23).

Molto più varie sono le condizioni nei girini sopravvissuti al 7° giorno. I più inibiti, come *N 3*, presentano condizioni non dissimili dagli embrioni del gruppo *M*, cioè totale assenza di ogni struttura pertinente al vestibolo orale o presenza di tubercolo impervio. Altri (*N 1*, *4*) hanno invece un'ampia apertura a contorni irregolari, il cui margine inferiore è fornito di papille, e di cui il superiore presenta una piccola sporgenza mediana: le due insellature laterali a questa rappresentano le fossette nasali (fig. 26). In altre parole, la parte prenasale della regione rostrale è inibita al punto che le narici confluiscono con la bocca. In al-

cuni casi la sporgenza mediana si prolunga a mo' di rostro, conferendo un curioso aspetto al capo del girino (figg. 27, 28). Non di rado la inibizione è asimmetrica, e una narice non è separata dalla cavità orale, mentre l'altra conserva un setto che la divide (fig. 28). Nei girini meno inibiti infine, il vestibolo orale è pressochè normalmente costituito, e le narici hanno una posizione quasi regolare. L'inibizione si manifesta, anzichè con un avvicinamento delle fossette nasali, come è stato spesso descritto in altri casi di inibizioni ottenute con il litio o altri reagenti, con una notevole riduzione in lunghezza dello spazio prenasale (fig. 29).



FIGG. 26–29.—Regione rostrale (dal dorso) dei girini *N 5*, *N 6*, *N 7*, *N 8*, di sette giorni. Diversi casi di inibizione dello sviluppo di questa parte. Questi girini sono assai meglio sviluppati che non quelli rappresentati nelle figg. 19–22, e hanno esaurito il tuorlo. (Cfr. tav. III, figg. 15–18.) (Ingr. 25 diam.)

Riassumendo si può dire che la parte apicale del capo si presenta diversamente malformata, a seconda del grado di inibizione. Questa può produrre (1) totale assenza del vestibolo orale e delle strutture annesse; in questi casi, generalmente, non si formano le narici; (2) assenza del vestibolo orale, ma presenza di rudimenti delle labbra, fuse in un tubercolo rivestito di papille; le fossette nasali sono assenti; (3) riduzione della regione rostrale dorsale e confluenza delle narici con il vestibolo orale; il labbro inferiore e talvolta anche la parte ventrale del becco corneo sono più o meno bene sviluppati; (4) accorciamento dello spazio prenasale e riduzione relativamente lieve del vestibolo; le strutture caratteristi-

che possono essere tutte presenti. Come si vede i risultati della inibizione sono sensibilmente diversi da quelli descritti per le larve al litio (cfr. Cotronei, 1921), che consistono piuttosto in una tendenza all'avvicinamento e alla fusione delle parti laterali lungo la linea mediana.

Branchie: Sono anch'esse notevolmente colpite dai processi inibitorî. Fra i girini di 4 giorni soltanto pochi presentano qualche ridottissimo filamento branchiale, mentre i controlli sono allo stadio rappresentato in fig. 13. Larve come *M 1, 3, 7* (figg. 14, 15, 16) muoiono senza che si sieno sviluppati i villi branchiali.

A 7 giorni si trovano, come già per il vestibolo orale, i più diversi gradi di sviluppo. Girini come *N 3* (fig. 21) non mostrano esternamente traccia di branchie. Altri, come *N 1, 2, 4* (figg. 19, 20, 22) hanno invece filamenti branchiali piccolissimi e atrofici. Girini meno inibiti si trovano invece, presso a poco, allo stadio di *Contr. M* (fig. 13) ma con filamenti branchiali meno sviluppati. In altri ancora, si va sviluppando l'opercolo, che, in quelli che sopravvivono più a lungo, si completa nei giorni successivi.

Dall'esame esterno degli ibridi di questo incrocio risulta come l'inibizione prodotta dall'ibridazione possa essere di grado molto diverso, e dia origine a malformazioni di differente gravità, che conducono alla morte in stadî più o meno precoci. Quando il disturbo non impedisce lo svolgersi normale della gastrulazione, gli embrioni appaiono colpiti specialmente nella regione cefalica, e le inibizioni sembrano localizzate particolarmente nella parte prenasale. Questa può essere ridotta fino a completa assenza del vestibolo orale, o presentarsi meno gravemente malformata. In alcuni girini ha aspetto pressochè normale e soltanto la brevità dello spazio prenasale rivela l'effetto di un processo inibitorio.

L'inibizione dell'accrescimento in lunghezza del corpo e della coda e l'intensità della curvatura dorsale, come pure lo sviluppo più o meno atrofico delle branchie sono strettamente correlati con il grado di intensità delle malformazioni cefaliche. Lo sviluppo degli organi adesivi sembra invece, in certa misura, indipendente dalla gravità dell'inibizione.

Non furono eseguite osservazioni accurate sui movimenti di questi girini. In generale sono molto più torpidi che non i controlli, e giacciono immobili sul fondo della vaschetta, finchè non vengano stimolati. I meno inibiti sono capaci di movimenti locomotorî coordinati, alcuni però eseguiscono movimenti di maneggio. I più gravemente

colpiti invece, se vengono stimolati, rispondono soltanto con movimenti di flessione unilaterale o a S.

2. *Il neurasse, la corda, gli organi di senso cefalici.*—Non molto v'è a dire sugli embrioni anulari, spina bifida, ecc., che rappresentano anomalie molto comuni in diverse condizioni sperimentali, e le cui caratteristiche sono state discusse in numerosi lavori. Le alterazioni che si incontrano negli embrioni che hanno gastrulato normalmente, hanno invece una fisionomia più caratteristica, e meritano perciò di essere brevemente descritte.

Embrioni di 4 giorni: L'embrione *M 6* (fig. 11 e tav. I, fig. 1) è un esempio dei casi di più intensa inibizione, che si trovano nelle culture al 4° giorno. Nè in questo nè in altri simili è possibile distinguere gli abbozzi del sistema nervoso e della corda. Questi embrioni, rivestiti di ectoderma qua e là raggrinzito in rughe e tubercoli, presentano organi adesivi ben sviluppati e funzionanti. L'interno è pieno di cellule cariche di granuli di tuorlo e non di rado di granuli di pigmento, fra le quali non è possibile distinguere alcun differenziamento organologico o istologico. Si tratta di embrioni anidiani, cioè di casi di aneurogenesi totale, che, evidentemente, derivano dai casi di mancata chiusura delle pieghe midollari, sopravvissuti fino al 4° giorno. Alcuni hanno il tappo vitellino sporgente e si sono liberati di una buona quantità di tuorlo, ma altri hanno il blastoporo completamente chiuso. Il tubercolo caudale dell'embrione *M 6*, e così pure quello cefalico, non contengono strutture differenziate.

Gli altri individui presentano gradi diversi di inibizione. Negli embrioni più gravemente colpiti, come *M 1, 3, 7* (figg. 14, 15, 16 e tav. I, fig. 2) lo sviluppo degli organi e il differenziamento istologico sono anche più arretrati che non nei controlli di due giorni (*Contr. J*, fig. 8). Nell'embrione *M 3*, ad esempio, l'intero prosencefalo è rappresentato da una massa piena, di forma irregolare e priva di cavità. Non vi si distingue alcun accenno di differenziamento nelle varie parti, nè si vedono gli abbozzi delle vescicole ottiche. Più caudalmente si osserva nell'interno del neurasse un lume assai irregolare. La parete dorsale della terza vescicola cerebrale è enormemente ispessita, mentre la ventrale è composta di un solo strato di cellule. Il midollo è pure provvisto, per tutto il suo percorso, di un lume irregolarissimo, ora ampio, in corrispondenza dei processi necrotici che colpiscono certi tratti delle pareti, ora ridottissimo. Delle otocisti, una è provvista di cavità, ma non ha accenno del dotto endolinfatico, l'altra invece è piena. La corda non presenta notevoli anomalie, se non l'arretrato stadio di differenziamento istologico.

In altri casi simili, in cui tutto il prosencefalo è privo di cavità, si può trovare accenno delle vescicole ottiche. In certi embrioni l'intensità dell'inibizione è alquanto più lieve (tav. I, fig. 3), i placodi olfattorî sono ben visibili e una cavità, più o meno irregolare, compare a livello delle vescicole ottiche, le quali non hanno ancora indotto nell'ectoderma la proliferazione lentogena. Il pavimento del dien-

cefalo appare spesso ripiegato a costituire un'ernia ventrale, indice dell'insufficiente turgore del ventricolo. Non di rado la cavità, che si trova a livello dei calici ottici, si oblitera più caudalmente e il cervello riappare massiccio. Frequentemente si osserva poi una notevole asimmetria, che può esprimersi con decorso interamente asimmetrico, o tortuoso del tubo midollare, o con notevole spostamento, in senso caudale o ventrale di una delle vescicole ottiche o delle otocisti. Spesso l'asimmetria è accompagnata da eterocronismo di sviluppo degli organi pari.

Le pareti dell'intero tubo neurale possono essere, nei varî tratti, anormalmente ispessite o sottili. I processi necrotici delle cellule nervose sono talvolta estesi ad ampie regioni, talora localizzati in focolai irregolarmente disposti lungo tutto il percorso, e più o meno estesi.

In conclusione gli embrioni di questo tipo presentano sintomi di una grave inibizione del sistema nervoso. Assai arretrati nello sviluppo rispetto ai controlli, possono presentare una totale inibizione del prosencefalo, per lo più accompagnata da mancato differenziamento delle vescicole ottiche e dei placodi olfattorî, o inibizione localizzata alla regione corrispondente al telencefalo, mentre il diencefalo e le vescicole ottiche hanno iniziato il loro differenziamento. In questi casi, generalmente, si trovano anche i placodi olfattorî. Le otocisti, di regola, sono meno colpite dal processo inibitorio e più o meno rudimentalmente differenziate in tutti i casi. Oltre a casi di notevole asimmetria nella posizione del neurasse o di alcune sue parti o nello spessore delle sue pareti, si incontrano irregolarità nella disposizione delle cavità (ineguaglianze anormali nel lume dei varî tratti, cavità separate da tratti massicci, ecc.) focolai necrotici più o meno estesi e localizzati in regioni diverse, lungo tutto il tubo neurale.

Assai diverso è l'aspetto degli embrioni del tipo di *M 4, 5*. Il differenziamento istologico è molto più avanzato e l'organogenesi è proceduta più regolarmente. Sono ad uno stadio paragonabile a quello dei *Contr. J.*

Nell'embrione *M 5* (tav. II, figg. 6–8) il tubo nervoso ha un lume singolarmente ampio rispetto all'esiguità delle sue pareti. Il telencefalo è ridotto soprattutto in lunghezza, e non vi è traccia della divisione nei due emisferi. I calici ottici non hanno indotto la formazione della lente, l'evaginazione infundibolare è appena accennata. Nella regione immediatamente retrostante al rombencefalo la continuità della parete ventrale del midollo si rompe e la corda si inserisce nella lacuna. In sezioni un po' più caudali, la corda occupa il canale ependimale, per poi tornare nuovamente nella posizione regolare. Forse questo fatto è da interpretarsi come una tendenza alla spina bifida.

L'embrione *M 4* (fig. 18) è alquanto più avanzato nello sviluppo, e mostra

TAVOLA I

FIG. 1.—Embrione *M 6* (fig. 11 nel testo) anidiano. Si vedono gli organi adesivi e alcuni stadî di alterazione delle cellule epidermiche.

FIG. 2.—Embrione *M 1* (fig. 15). La sezione incontra una otocisti e l'opposta vescicola ottica, di cui, in sezioni più rostrali, si vede il peduncolo. Cervello massiccio; in sezioni più cefaliche è presente la cavità.

FIG. 3.—Embrione *M 8*. Sezione a livello delle vescicole ottiche, che non presentano ancora la introflessione. Pavimento del diencefalo incurvato ventralmente.

FIG. 4.—Embrione *M 4* (fig. 18). Sezione a livello della parte caudale del telencefalo.

TAVOLA II

FIG. 5.—Embrione *M 4* (fig. 18 nel testo). La sezione interessa un calice ottico e mostra la cavità dilatata del diencefalo.

FIG. 6.—Embrione *M 5*. La sezione interessa la parte rostrale del diencefalo, enormemente dilatata, e taglia anche i placodi olfattorî.

FIG. 7.—Embrione *M 5*. Sezione nella regione del midollo allungato, che mostra come la corda si insinua nel canale ependimale.

FIG. 8.—Embrione *M 5*. Sezione a livello delle vescicole ottiche, non ancora introflesse. Cavità cerebrale e faringea molto dilatate.

TAVOLA I

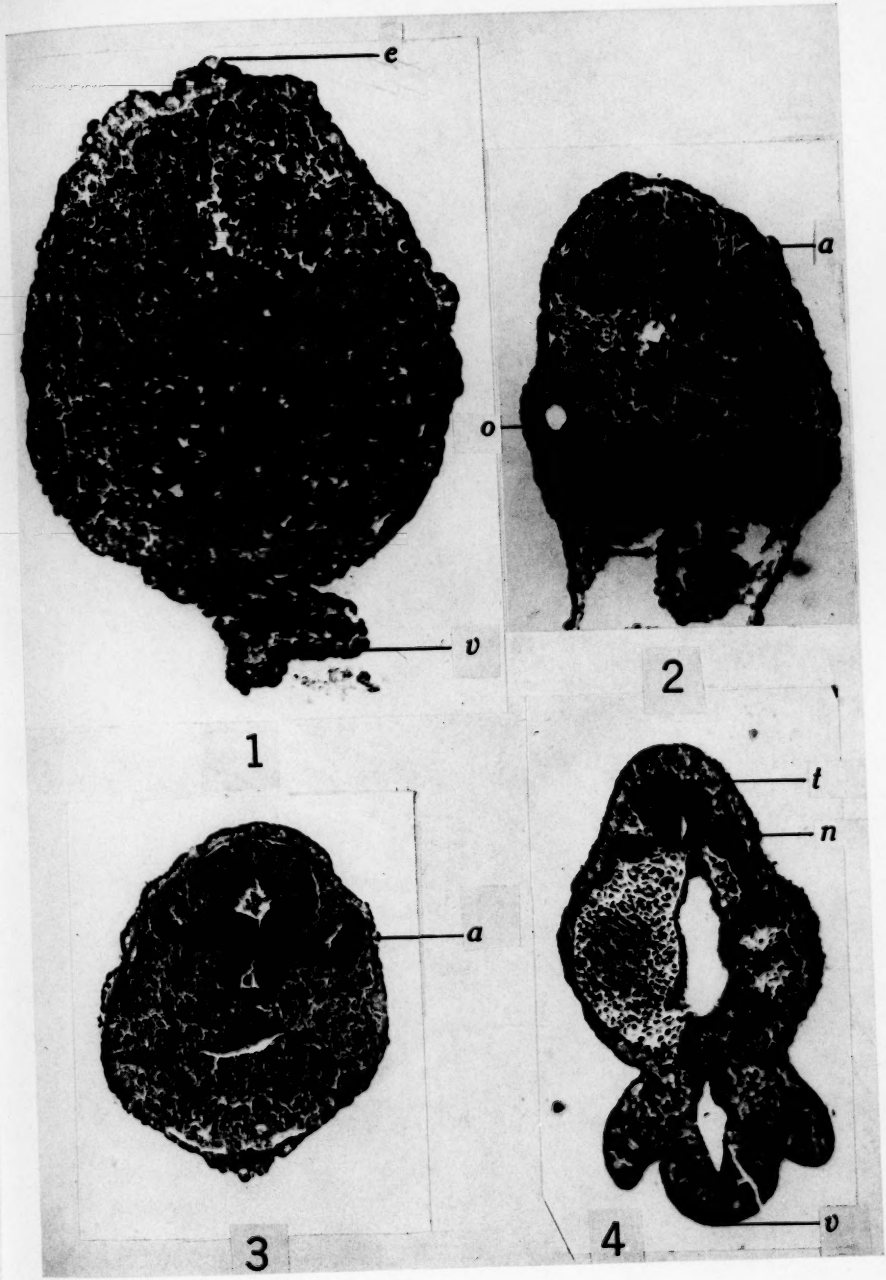
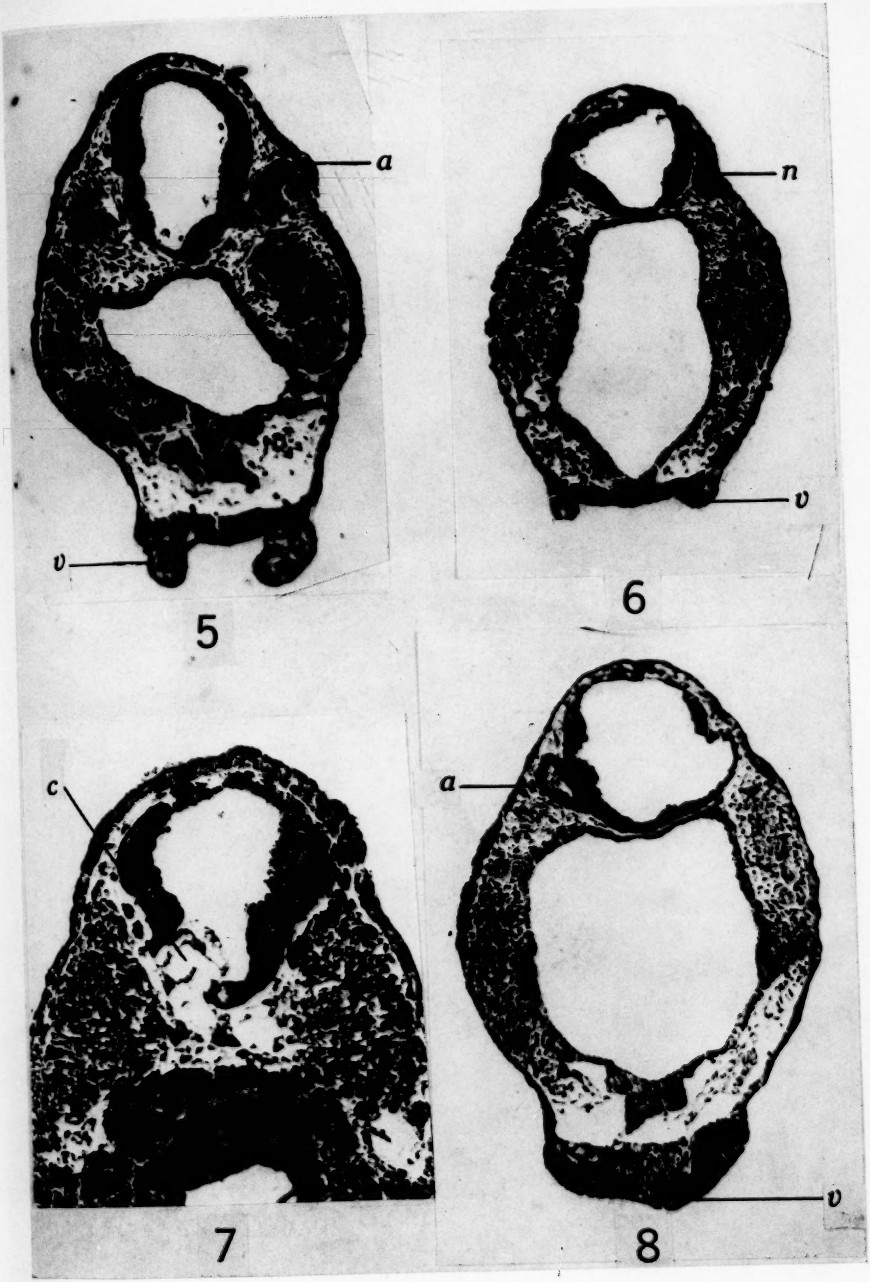


TAVOLA II



anch'esso una considerevole inibizione del telencefalo (tav. I, fig. 4, tav.II, fig. 5). I placodi olfattorî sono tuttavia ben differenziati e la proliferazione delle loro cellule si spinge verso la cavità faringea. Il diencefalo ha aspetto quasi normale, ma è di dimensioni assai ridotte, specialmente nella sua parte più craniale, ed ha il pavimento sottilissimo. Gli abbozzi oculari si sono avvicinati all'ectoderma e vi hanno indotto la formazione della lente. Il recesso infundibolare è piuttosto cospicuo, relativamente alle dimensioni del diencefalo. Le otocisti mostrano la formazione dell'abbozzo del dotto endolinfatico. Il tubo midollare ha poi un decorso piuttosto regolare ed è provvisto di un ampio canale ependimale. Tutto il neurasse ha pareti estremamente sottili in cui abbondano minuti centri di necrosi. Il differenziamento delle fibre non ha ancora avuto luogo.

Gli embrioni del tipo di *M 4, 5* sono dunque notevolmente meglio sviluppati di quelli del gruppo precedente e più avanzati nel differenziamento istologico e organologico. L'inibizione, per quanto riguarda la morfogenesi, sembra limitata soprattutto al telencefalo, mentre le altre parti del sistema nervoso ne sono assai meno gravemente colpite. L'effetto dell'inibizione si manifesta poi nell'esiguità delle pareti di tutto il tubo neurale, a cui spesso corrisponde un'ampiezza sproporzionata delle cavità, nel ritardato differenziamento degli organi, e in alcune anomalie di posizione e di struttura di alcune parti, analoghe a quelle del gruppo precedente.

Altri embrioni presentano condizioni simili a quelle dei suddescritti, e alcuni manifestano inibizione di intensità anche minore, i cui effetti appariranno più chiari dall'esame degli embrioni più avanzati.

Embrioni di 7 giorni: Alcuni degli embrioni che sopravvivono fino al 7° giorno non sono sensibilmente più progrediti di quanto lo siano quelli del gruppo *M*, così p. es. *N 3*. Molti, meno inibiti, presentano un ulteriore differenziamento organico e si trovano presso a poco allo stesso stadio dei controlli di 4 giorni (*Contr. M*, fig. 13) e alcuni sono anche un po' più avanzati.

L'embrione *N 2* (fig. 19) presenta la parte anteriore del telencefalo ridotta ad una masserella di cellule, impari, in cui non v'è traccia di ventricoli. In sezioni più caudali questa viene a disporsi dorsalmente al talamo. Nella sezione rappresentata in tav. III, fig. 9, si vedono nel cervello tre cavità, di cui la più dorsale rappresenta la parte caudale del telencefalo, quella di mezzo il terzo ventricolo, e quella inferiore, probabilmente, la regione infundibolare, le cui pareti sono notevolmente ridotte e addossate alla corda. A destra la sezione incontra una vescicola ottica, con la lente; il *tapetum* è ben riconoscibile, ma non sembra esservi accenno di un ulteriore differenziamento istologico della retina. A sinistra invece la sezione taglia il territorio olfattorio: l'asimmetria di tutta la regione cefalica è infatti notevolissima. Le vescicole otiche presentano pareti molto sottili e cavità assai ampia; le evaginazioni del dotto endolinfatico e dei canali semicirco-

TAVOLA III

FIG. 9.—Embrione *N 2* (fig. 19 nel testo). La sezione interessa un occhio e l'opposto placode olfattorio, la regione più caudale del telencefalo e il diencefalo. Il cuore è tagliato al punto di transizione fra la parte ventricolare, massiccia, e la auricolare, cava e a pareti sottili.

FIG. 10.—Embrione *N 4* (fig. 20). Sezione interessante il telencefalo, che ha posizione dorsale al diencefalo. Cuore massiccio (regione ventricolare).

FIG. 11.—Embrione *N 4*. Sezione più caudale, a livello degli occhi.

FIG. 12.—Embrione *N 4*. Sezione a livello delle otocisti. Si vede la parte cefalica, dilatata, del pronefro.

FIG. 13.—Embrione *N 1* (fig. 22). La sezione interessa la parte apicale del telencefalo e una parte del diencefalo, con una vescicola ottica addossata.

FIG. 14.—Embrione *N 1*. Sezione più caudale, che incontra un occhio e un'otocisti.

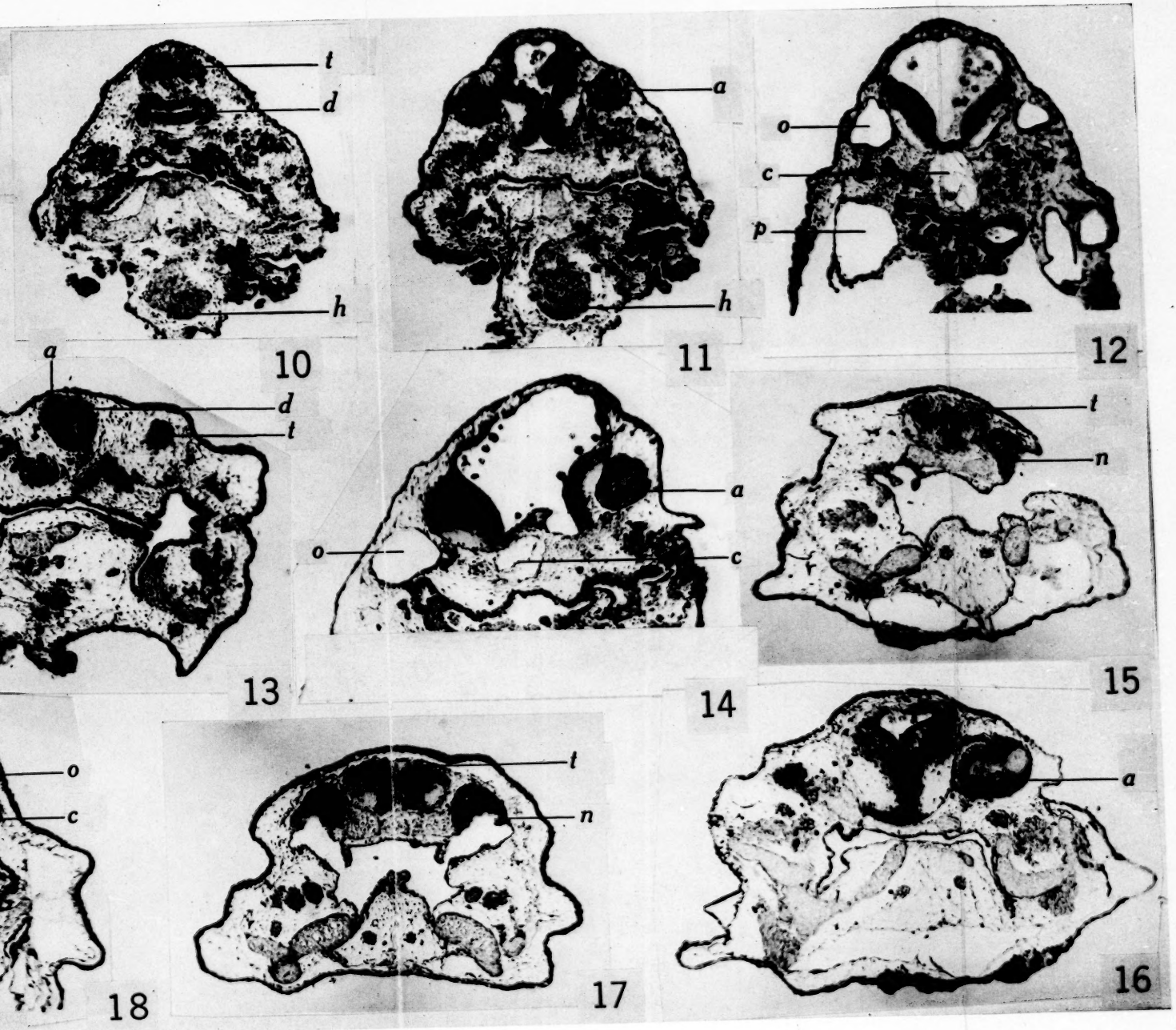
FIG. 15.—Embrione *N 7* (fig. 28). Sezione nella regione rostrale, che mostra la riduzione degli emisferi cerebrali.

FIG. 16.—Embrione *N 7*. Sezione più caudale, a livello degli occhi.

FIG. 17.—Embrione *N 8* (fig. 29). Sezione nella regione rostrale, che mostra gli emisferi cerebrali meglio sviluppati che non nell'embrione precedente.

FIG. 18.—Embrione *N 8*. Sezione a livello delle otocisti. Cuore dilatato, a pareti sottilissime (regione auricolare).

TAVOLA III



lari sono appena accennate. Il quarto ventricolo ha lume molto irregolare, in certi punti le pareti opposte vengono a contatto e si fondono, e si determina così la formazione di piccole cavità accessorie. Più caudalmente, la cavità del quarto ventricolo diviene amplissima, la parete ventrale è sottile e distesa, la tela corioidea, enormemente sviluppata, forma un'appendice a sacco rivolta caudalmente. Il midollo spinale ha un diametro esiguo e va estremamente assottigliandosi verso la coda. Si distinguono i gruppi di cellule destinati a formare i gangli cranici e spinali. La corda è ipertrofica in tutta la sua estensione e non presenta che una esilissima guaina scheletogena.

Condizioni simili si trovano in *N 4* (fig. 20). Telencefalo ridottissimo e sovrastante il diencefalo, di cui si vede, in tav. III, fig. 10, la parte apicale, appiattita. La fig. 11 (tav. III), a livello delle vescicole ottiche, mostra il lume irregolarissimo della cavità del terzo ventricolo. La fig. 12 (tav. III) in corrispondenza delle otocisti, mostra l'ampiezza del quarto ventricolo e lo sviluppo della tela corioidea. Anche qui il midollo è di diametro assai ridotto e la corda ipertrofica.

L'embrione *N 1* (fig. 22) presenta una considerevole asimmetria, pur avendo sviluppati tutti gli organi principali. La fig. 13 (tav. III) rappresenta una sezione nella regione rostrale e mostra a sinistra una vescicola ottica, addossata alla parete del diencefalo. A destra si vede invece la porzione craniale del telencefalo, ridotta ad un sottile cordone impari e pieno. In una sezione più rostrale si può vedere a sinistra un rudimento del placode olfattorio. In sezioni più caudali a quella raffigurata si osserva come il telencefalo si disponga dorsalmente al diencefalo, come avviene negli altri embrioni. Si incontra poi il placode olfattorio destro e (tav. III, fig. 14) l'occhio destro, allo stesso livello della otocisti sinistra. Anche qui nella regione del quarto ventricolo la cavità, assai irregolare, è suddivisa in piccoli canali accessorî, e la tela corioidea, ipertrofica, presenta un'appendice sacciforme caudale.

In tutti questi casi la sostanza grigia e la bianca sono differenziate, ma, come si vede dalle microfotografie, lo spessore della parete di tutto il tubo neurale è oltremodo esiguo.

L'inibizione ha dunque colpito fortemente tutto il neurasse. Più gravemente affetto ne è il telencefalo, specialmente nella regione dei lobi olfattorî. Il suo sviluppo in lunghezza pare impedito, tanto che assume una posizione dorsale al diencefalo, che, a questo stadio di differenziamento è del tutto anormale. L'alterazione delle rimanenti parti dell'encefalo è grave, ma meno profonda. Gli occhi sono generalmente sviluppati, ma hanno piccole dimensioni, e, per la brevità del peduncolo, sono quasi aderenti alla parete cerebrale. Ancorchè si osservi una indubbia riduzione del pavimento del diencefalo, che si manifesta spesso con l'insufficiente sviluppo dell'infundibolo, non si verifica un avvicinamento delle vescicole oculari tale da produrne

la fusione totale o parziale: nessun caso di ciclopia è stato mai osservato nei diversi esperimenti.

Varî disturbi colpiscono le altre parti del cervello, e le pareti del tubo neurale appaiono ridotte, per tutta la sua estensione, mentre la corda è ipertrofica. Una notevole asimmetria nello sviluppo e nella posizione degli organi di senso cefalici si manifesta non di rado.

Fra i girini meno inibiti e più avanzati nello sviluppo, che hanno per lo più iniziato la formazione dell'opercolo, alcuni presentano notevoli malformazioni nella regione rostrale, altri invece non mostrano che un accorciamento della regione prenasale, come risulta dalle fig. 26–29. A questi fenomeni corrispondono diversi gradi di inibizione del telencefalo, mentre il resto del neurasse è pressochè normale.

Nel girino *N 7* (fig. 28) il corno frontale contiene due trabecole cartilaginee, i *cornua trabeculae* notevolmente avvicinati; le cartilagini labiali superiori o suprarostrali non si sono sviluppate affatto. Il telencefalo appare assai ridotto (tav. III, fig. 15) ma tuttavia occupa la posizione normale; gli emisferi non sono distinguibili e le cavità dei due primi ventricoli sono completamente assenti. Il diencefalo, a lume ridottissimo nella sua parte anteriore, appare poi quasi normale. Gli occhi sono piccoli e la cavità del vitreo è del tutto assente (tav. III, fig. 16); la retina è istologicamente molto meglio differenziata che non negli embrioni fin qui considerati. Una lieve asimmetria si manifesta nella posizione degli occhi e nelle loro dimensioni: il destro è più accostato al cervello e più grosso, e anche la parete cerebrale è in questo tratto, più sviluppata a destra. Il rimanente dell'encefalo non è quasi distinguibile da quello di un embrione normale. Il differenziamento meno progredito che nei controlli coetanei, le pareti del neurasse meno sviluppate e alcune zone necrotiche e globuli di pigmento che s'incontrano qua e là anche nelle sezioni più caudali, palesano gli effetti dell'inibizione. La corda è ipertrofica, ma meno che nei casi finora considerati; il midollo spinale ha anche qui un diametro piuttosto ridotto.

Anche meglio sviluppato è il girino *N 8* (fig. 29). Qui le cartilagini soprarostrali sono riconoscibili, benchè di dimensioni assai ridotte. Il telencefalo è assai più regolare (tav. III, fig. 17), gli emisferi sono distinti e provvisti di ventricoli, soltanto il lobo olfattorio è assai ridotto. Il midollo spinale è un poco più sviluppato che in *N 7*, e la corda assai meno ipertrofica (tav. III, fig. 18). Alcuni altri girini sono anche meno colpiti, tanto che, come si è detto, esternamente sono quasi indistinguibili dai normali.

In questi casi l'inibizione dei processi morfogenetici è anche più localizzata nella regione anteriore, olfattoria del telencefalo. Nei casi più gravi di questa categoria, le cavità dei due primi ventricoli cerebrali sono assenti e la formazione delle cartilagini labiali superiori è impedita, così che le narici confluiscono con la bocca. Nei casi meno

gravi soltanto la regione apicale degli emisferi è inibita, i ventricoli sono presenti e i girini hanno aspetto poco diverso dai controlli più giovani.

3. *Cuore e sistema circolatorio.*—Il cuore è uno degli organi più gravemente colpiti dalla inibizione e i vasi e le branchie risentono del suo insufficiente sviluppo e sono perciò anch' essi ridotti o malformati. Le condizioni sono simili a quelle descritte da Reagan e Thorington (1915) negli ibridi interspecifici di Teleostei e da Reagan (1915) negli embrioni trattati con reagenti chimici.

Nei meglio sviluppati degli embrioni di 4 giorni (*M 4, 5*) l'abbozzo cardiaco si presenta come un sottile tubicino, non di rado schiacciato, a decorso irregolare, rivestito di cellule endoteliali e sospeso per un esile mesocardio nella cavità pericardica, la quale è generalmente assai più dilatata che negli embrioni normali. I controlli coetanei hanno già il cuore ben differenziato, sangue ricco di emazie e branchie sviluppate.

Negli embrioni di 7 giorni più gravemente inibiti (*N 1, 2, 4*) il cuore è rappresentato da un cordone ricurvo a S, solido in tutta la parte più craniale (tronco arterioso e ventricolo). Nella parte caudale invece (orecchiette, seno venoso) le pareti sono oltremodo sottili e circondano una cavità abbastanza ampia, indivisa (tav. III, fig. 9–11). Il differenziamento delle fibre muscolari cardiache è evidentemente alterato ed è chiaro che un simile organo non può funzionare. Le aorte sono rappresentate da due tubicini esilissimi, a malapena riconoscibili fra il mesenchima che le circonda, e non è possibile vedere alcuna loro connessione con le branchie. L'ematopoiesi è quasi totalmente inibita, soltanto in alcuni embrioni si trova qualche raro corpuscolo sanguigno.

Negli embrioni meno gravemente colpiti, come *N 7, 8*, il cuore non è così profondamente alterato, benchè si mostri considerevolmente inibito rispetto ai controlli. Tronco arterioso, ventricolo, atrî sono ben riconoscibili e hanno acquistato le normali relazioni di posizione, ma le pareti sono sottili e presentano indubbî segni di debilità (tav. III, fig. 18). Le aorte e le vene cardinali sono pure riconoscibili, e gli archi aortici hanno stabilito le connessioni con le branchie.

Le emazie, sempre in numero assai ridotto rispetto ai controlli, hanno aspetto molto anormale, per la forma e le dimensioni irregolari, e perchè hanno nuclei talvolta enormi, talvolta picnotici e frammentati. Non è raro il fenomeno segnalato da O. Hertwig per i girini irradiati, di ampie ipostasi nei vasi o in grosse lacune, che appaiono pieni di emazie, mentre il cuore e gli altri vasi ne sono privi. Si trovano per lo più tutte dalla stessa parte dell'embrione e, secondo l'intepretazione del Hertwig, sono dovute alla debolezza del movimento di propulsione del cuore e al fatto che i girini giacciono quasi sempre immobili su di un fianco. Le emazie si dispongono perciò secondo la gravità, raccogliendosi in certi punti e dilatando il lume dei vasi, donde, forse per lacerazione della parete endoteliale, si riversano anche nella cavità del corpo.

Anche nei casi in cui l'inibizione sembra più lieve, a giudicare dalla forma esterna e dal grado di riduzione del telencefalo, l'esilità e l'aspetto anormale del cuore e dei vasi e lo scarso numero di globuli rossi ci dicono che il sistema circolatorio è gravemente alterato e il suo funzionamento stentato e insufficiente. Dev'essere questa una causa, e non l'ultima, della scarsa vitalità di tutti i girini, compresi quelli apparentemente poco diversi dai normali.

4. *Gli altri sistemi.*—Le modificazioni che subiscono gli altri sistemi sono, per quanto si riferisce alla morfogenesi, di minore entità. Il loro grado di differenziamento dipende, naturalmente, dallo stadio di sviluppo che l'embrione può raggiungere, ma in ogni caso non sembrano questi i principali responsabili della sua morte.

L'epitelio dell' *intestino* sembra piuttosto suscettibile alla inibizione, a giudicare dal fatto che, a somiglianza del sistema nervoso mostra qua e là zone necrotiche e pigmento sparso fra le cellule. Il canale digerente si differenzia tuttavia abbastanza regolarmente, negli embrioni meno colpiti, e presenta normale sviluppo delle cavità. Nei pochi girini che raggiungono stadi più avanzati e che esauriscono le riserve vitelline, si trovano qua e là nel lume scarsi frammenti di alghe e di altro materiale ingerito. La parte stomodeale si sviluppa soltanto in quegli embrioni che non presentano gravissima inibizione della regione rostrale, in questi, come si è detto, la formazione della bocca è impedita e l'intestino non comunica con l'esterno. Anche in questi casi, tuttavia, si può osservare la formazione degli abbozzi delle tasche branchiali a spese dell'entoderma faringeo, rappresentati da gruppi di cellule massicci, che, in questi embrioni, non si scavano. Non di rado il lume intestinale, nella regione faringea, è insolitamente ampio (tav. II, fig. 6, 8). Nelle larve meno gravemente affette l'invaginazione stomodeale si unisce con l'evaginazione della faringe, le tasche branchiali si sviluppano ulteriormente, e si stabilisce anche la comunicazione del retto con l'esterno.

Il fegato è generalmente riconoscibile, e non dà segni di grave alterazione, nei girini come *M 4, 5*, e in tutti quelli che arrivano fino al 7° giorno.

Lo stesso si può dire del *pronefro*, le cui cellule cubiche sono però alquanto striminzite e circondano talvolta tubuli insolitamente grandi. Nei girini del 7° giorno il pronefro comunica regolarmente con la cavità celomatica, ma talvolta presenta una considerevole dilatazione nella parte cefalica (tav. III, fig. 12).

I *somiti* sembrano formarsi regolarmente, ma lo sviluppo della muscolatura segmentale risente gravemente gli effetti dell'alterazione patogena. Lungi dall'acquistare le dimensioni normali, i miotomi sono tanto più ridotti quanto più grave è l'alterazione che colpisce tutto l'embrione. Probabilmente il numero dei somiti non è inferiore al normale (non furono eseguiti conteggi esatti) ma certo il differenziamento dei muscoli è inibito almeno quanto quello del cuore.

Gli abbozzi delle *gonadi* sono visibili nei meno malformati fra gli embrioni di 7 giorni, ma la loro esiguità, l'irregolarità dei contorni, e l'abbondante pigmento

che si trova fra le cellule, rivelano che hanno gravemente risentito gli effetti della malattia che colpisce questi ibridi.

Meno suscettibili sembrano invece i tessuti di *sostegno* e di riempimento. La corda, come già si disse, è in genere ben differenziata, turgida e spesso ipertrofica. Il mesenchima larvale ha aspetto quasi normale, e della cartilagine si può dire che è differenziata normalmente, in quelle larve che hanno raggiunto uno stadio sufficientemente avanzato. Soltanto nella regione rostrale si può constatare l'inibizione dello sviluppo di qualcuna delle cartilagini: può aversi l'assenza dell'abbozzo o il suo incompleto sviluppo e forma irregolare, specialmente nei mostri asimmetrici. Deve trattarsi, evidentemente, di un fenomeno secondario, determinato dal mancato sviluppo di altri organi.

C. L'ISTOGENESI

I tessuti embrionali e larvali degli Anfibî sono un materiale che assai poco si presta all'indagine istologica e citologica, e la tecnica usata per la fissazione e colorazione degli embrioni non ci consente uno studio molto accurato in questo senso. È tuttavia opportuno qualche cenno sommario sul grado di alterazione subita dai varî tipi di cellule, perchè ce ne dimostra il diverso grado di suscettibilità. Ed è bene tener presenta il fatto, sottolineato dal Ranzi (1928) per gli embrioni di Cefalopodi trattati con i sali di litio e dal Pasquini (1931*a*) per gli embrioni di Anfibî irradiati, che può esistere una certa indipendenza fra i processi morfogenetici e gli istogenetici. Benchè esista "una netta relazione d'interdipendenza fra deviazioni e inibizioni della normale morfogenesi e le alterazioni del differenziamento istologico" è possibile, con dosi opportune applicate negli stadî meno suscettibili, dar luogo ad alterazioni dell'istogenesi non accompagnate da malformazioni organologiche (Pasquini, 1931*a*). Lo stesso fenomeno si può riconoscere in questi ibridi, dove un certo grado di alterazione delle cellule è tuttavia compatibile con un differenziamento morfogenetico pressochè normale.

Il differenziamento istologico, in questi embrioni, va di pari passo con il differenziamento organico e procede nell'ordine in cui avviene nell'embriogenesi normale: l'epidermide, il tessuto nervoso e la corda sono i primi a differenziarsi, poi il tessuto muscolare e cardiaco, il tessuto escretore, l'intestino, e infine, la cartilagine. Man mano che procedono nel differenziamento istologico le cellule si liberano dalle riserve vitelline che posseggono, talchè infine ne risultano del tutto

prive. È questo, mi pare, un fatto contrario alla teoria del Hertwig, che verrà esposta più tardi (pag. 389).

L'*epidermide* presenta, come si disse, una particolare tendenza a rompersi, specialmente nella regione ventrale del tronco. Le sue cellule manifestano pochissima forza di coesione e molte se ne vedono che fanno ernia all'esterno e vengono poi espulse (tav. I, fig. 1). Su tutto il corpo degli embrioni si osservano grinze e tubercoli, costituiti da gruppi di cellule gonfie e in procinto di rompersi, o talvolta da più strati di cellule. Il primo stadio di alterazione è rappresentato da un rigonfiamento del corpo cellulare e da una dispersione per il plasma dei granuli di pigmento, che normalmente sono addensati alla periferia e specialmente verso la parte distale della cellula. Il nucleo sembra poi dissolversi e la cellula si muta in un otricolo trasparente, in cui le sole strutture riconoscibili sono i granuli di pigmento; indi scoppia o viene espulsa. In altri punti invece lo strato epidermico appare sottile, come disteso da un'eccessiva tensione. Negli embrioni meno gravemente colpiti l'epidermide ha un aspetto più normale, ma è sottile, fragilissima. Quasi sempre si trovano, anche qui, tubercoli che risultano costituiti da zone necrotiche ispessite e fortemente pigmentate, in cui le cellule vanno disfacendosi. La dispersione del pigmento per il corpo cellulare e l'abbondante perdita di cellule che si verificano negli embrioni più colpiti spiegano il colore chiaro dell'epidermide di questi mostri, in cui la produzione del pigmento non è certamente inferiore a quella dei normali.

Il *tessuto nervoso* presenta, fin da stadî molto precoci, gravi sintomi di alterazione, riconoscibili all'abbondante deposito di pigmento, in minutissimi granuli, che stanno fra le cellule o nel loro interno. Alcune di esse sono molto più cariche delle altre e vengono poi espulse nel canale ependimale (tav. I, fig. 3; tav. III, fig. 9, 11, 12, 14, 16) o all'esterno, fra gli altri tessuti, come globuletti scuri, in cui talvolta è riconoscibile un nucleo picnotico. Alcune cellule che si trovano nel canale ependimale hanno nuclei deformati, ma non sono così fortemente pigmentate. Questa espulsione di cellule si osserva fin dai primi stadî della formazione del tubo neurale e si ritrova in tutti gli stadî successivi; ciò dimostra che le varie cellule si ammalano a periodi diversi del differenziamento. L'intensità della pigmentazione sembra essere proporzionale al grado di alterazione del tessuto. Tutto il tubo nervoso è colpito, ma spesso i processi necrotici sembrano localizzati in certe aree, irregolarmente disposte e di diversa ampiezza. Tali aree necrotiche e grossi accumuli vescicolari di pigmento sono particolarmente frequenti là dove l'inibizione è più grave, quindi nel telencefalo e nella sua parte apicale. Il pigmento si dispone di preferenza intorno ai nuclei, e nei casi in cui è avvenuto il differenziamento delle fibre, al limite fra la sostanza grigia e la bianca. Le fibre ne sono invece quasi prive, e i pochi granuli che vi si trovano sembrano essersi originati altrove. Anche negli embrioni normali è presente una certa quantità di pigmento disposto soprattutto intorno ai nuclei, nel tessuto nervoso, e assente nelle fibre.

L'impressione che si riceve dall'esame delle regioni più inibite è la mancanza quasi totale di corpo cellulare: i nuclei sono addossati talmente che in certi punti

i loro limiti sono assolutamente indistinguibili (in sezioni di 7 μ) e formano un'unica massa colorata in azzurro dalla ematossilina. Anche nelle formazioni derivate dal neurasse, come occhi e gangli, l'alterazione dei tessuti presenta gli stessi sintomi. Meno intensa è invece l'espulsione di cellule dagli organi formati dai placodi (olfattori e acustici); anche qui tuttavia si scorgono i sintomi di gravi condizioni patologiche, soprattutto per la esilità degli strati cellulari. Da tutti questi fenomeni si ricava l'impressione che il tessuto nervoso sia, fra tutti, il più gravemente colpito, per tutta la sua estensione. La parte craniale appare, anche istologicamente, più gravemente alterata del resto.

Colpito in modo assai grave è anche il tessuto *muscolare*. Le fibre sono generalmente gracili, con grossi nuclei, che talvolta fanno ernia attraverso il sarcolemma. La striatura è a malapena riconoscibile in alcune. Abbondanti granuli di pigmento sono deposti all'interno, specialmente intorno ai nuclei, e fra fibra e fibra. Queste hanno spesso sezione irregolare e decorso serpeggiante, e sono lassamente unite in esili fasci. Le fibre cardiache manifestano fenomeni analoghi, forse anche più intensi; sono, per lo più, strettamente avvicinate, poco colorabili con l'eosina e cariche di pigmento, che si deposita specialmente alla loro periferia.

Le cellule dell'*epitelio intestinale* si differenziano abbastanza regolarmente e costituiscono un epitelio cilindrico, con nuclei basali, nel quale sono riconoscibili le cellule mucose. La formazione di pigmento, molto meno abbondante che nei tessuti fin qui ricordati, è tuttavia considerevole e rivela una certa sensibilità dell'epitelio intestinale. La tonaca muscolare è estremamente sottile.

Le cellule del fegato sembrano molto meno suscettibili; negli embrioni più avanzati sono normalmente differenziate, come nei controlli dello stesso stadio e disposte nel modo caratteristico, senza pregiudizio per le formazione dei canalicoli e delle lacune. Nei vasi epatici si trovano poche emazie.

Le cellule del *mesenchima* offrono un aspetto assai regolare, sia individualmente che per la disposizione di questo tessuto nelle varie regioni dell'embrione. Qua e là si osservano accumuli di pigmento, talvolta stratificato intorno agli organi, che probabilmente proviene, almeno in parte, da altri tessuti. Non mancano tuttavia, anche nel mesenchima, cellule malate, con nuclei picnotici e in via di disfacimento.

Il tessuto *cordoide* è normalmente differenziato, anzi ipertrofico: le grosse cellule turgide, dalle sottili pareti, lungo le quali è deposto qualche granulo di pigmento, sembrano avere risentito assai poco gli effetti della alterazione. Come già si è detto, il loro grado di sviluppo è inversamente proporzionale al grado di inibizione dell'embrione.

Pure le cellule *cartilaginee* non differiscono dalle normali, nè per forma nè per posizione nella sostanza fondamentale. La produzione di pigmento in questo tessuto è praticamente nulla. Soltanto in alcuni punti, dove lo sviluppo normale della cartilagine è impedito, probabilmente per condizioni esterne ad essa, le cellule appaiono piccole, avvicinate, deformi, e la sostanza intercellulare non assume l'estensione regolare.

Da questi cenni sommarî si vede come i diversi tessuti risentono l'azione patogena con intensità molto differente. La manifestazione più imponente dello stato patologico (oltre il mancato o insufficiente sviluppo delle forme) è la formazione del pigmento bruno scuro, che si raccoglie nelle cellule e negli spazî intercellulari. In uno stesso tessuto, le diverse cellule sembrano essere diversamente colpite dall'alterazione patologica, e mentre alcune muoiono fin dai primi stadî, altre vengono espulse successivamente, per tutta la durata della vita dell'individuo. È evidente inoltre che ad una malformazione nella morfogenesi corrisponde un'alterazione istologica; tuttavia, quando quest'ultima non sia molto grave, lo sviluppo degli organi può non essere seriamente compromesso.

Disponendo i tessuti in ordine di decrescente suscettibilità, si ottiene la seguente serie: in primo luogo il tessuto nervoso e l'epidermide, poi il tessuto muscolare cardiaco e somatico, in terzo luogo l'intestino e i suoi derivati, e da ultimo i tessuti di sostegno e di riempimento: corda, mesenchima e cartilagine.

V. L'INCROCIO BUFO VULGARIS ♀ × B. VIRIDIS ♂

L'embriogenesi di questi ibridi non è stata studiata completamente, perchè lo scopo principale era di condurre i girini alla metamorfosi, per studiarvi il comportamento dei caratteri ereditarî. Alcune osservazioni, che furono fatte, presentano tuttavia un certo interesse, e intendo perciò riferirle, così incomplete como sono.

Anche in questo incrocio la percentuale delle uova che si orientano è simile a quella dei controlli (uova di *B. vulgaris* normalmente fecondate), e la segmentazione avviene presso a poco con la stessa velocità. La gastrulazione avviene in modo molto più normale che non nell'incrocio precedente, benchè in un certo numero di uova appaia alquanto ritardata. Qualche anomalia grave e precoce, dipendente da disturbi nella gastrulazione, si verifica anche qui, ma è limitata a pochi embrioni. Come già abbiamo avvertito, anche nella fecondazione normale se ne incontra, generalmente, qualche caso.

Lo sviluppo procede poi abbastanza regolarmente, ma non si può dire assolutamente identico a quello dei controlli. Come dimostrano le figg. 30–36, gli embrioni ibridi hanno, in stadî precoci, un aspetto alquanto anormale, che si manifesta soprattutto con la protuberanza

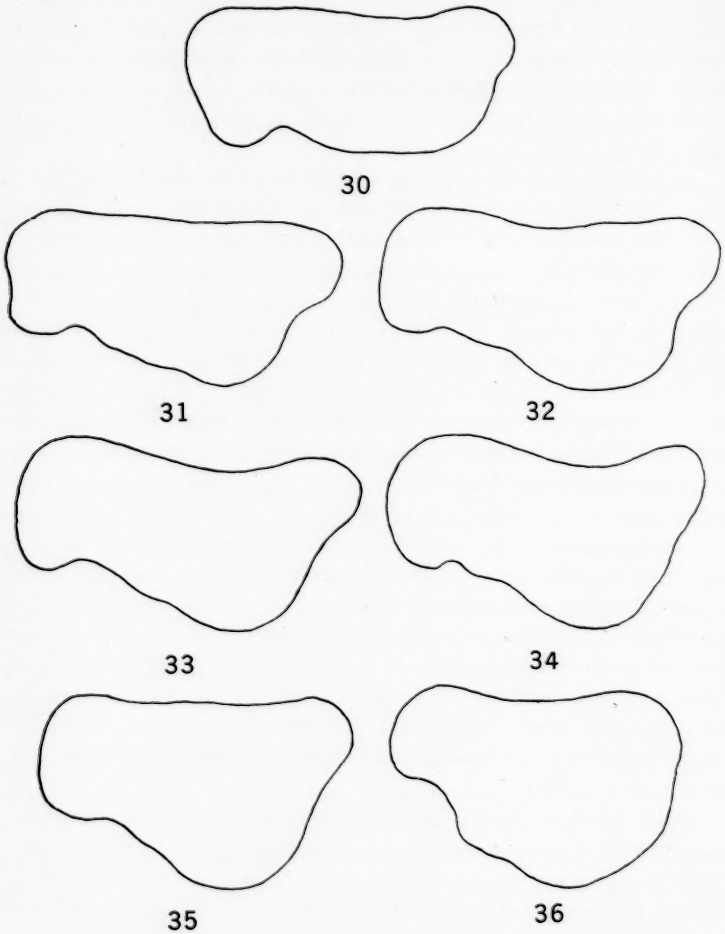
che la massa vitellina fa ventralmente. Alcuni embrioni appaiono inoltre un po' arretrati rispetto ai controlli e ai proprî confratelli. Il colore è eguale e quello dei controlli, la vitalità e la mobilità pure. In sezione, a questo stadio, si osserva soltanto un certo eterocronismo nello sviluppo dei diversi individui, che conferma quanto già indicava l'osservazione esterna. Probabilmente ai fenomeni accennati non è estranea la quantità eccessiva di tuorlo, e forse una certa difficoltà nella sua digestione, ricordate già da altri autori, soprattutto per i Pesci.

In seguito, mentre quasi tutti gli ibridi procedono regolarmente, alcuni cominciano a manifestare sintomi di idropisia, curvatura dorsale (non mai, però, così pronunziata come nell'incrocio reciproco) e nanismo. Questi muoiono, generalmente prima di avere raggiunto il 15°–20° giorno. Rivelano quasi tutti un grado lievissimo di inibizione del telencefalo, che li rende paragonabili ai meglio sviluppati dei girini ottenuti nel precedente incrocio, e che non provoca in genere deformazioni cospicue della regione rostrale. Il colore più pallido e la presenza di tubercoli sull'epidermide, nonchè l'idropisia, sono fenomeni che hanno comuni con i girini dell'incrocio reciproco. Hanno dimensioni molto variabili, da vicine alla media a molto inferiori, e i più piccoli sono anche i più arretrati nel differenziamento organico. La somiglianza di alcuni di questi individui con quelli ottenuti con la partenogenesi per puntura o per irradiazione, e le dimensioni esigue, dànno il sospetto che, in alcuni casi almeno, si tratti di embrioni aploidi, partenogenetici. Questa supposizione non fu potuta confermare con alcune misure di nuclei che furon fatte, ma la possibilità non deve perciò essere esclusa. Quasi tutti questi embrioni inibiti, tuttavia, sono certamente diploidi, e presentano, evidentemente, fenomeni simili ai meno gravemente colpiti degli ibridi reciproci.

Gli altri embrioni, per la massima parte, presentano invece sviluppo normale. Sono forse, in media, leggermente più piccoli dei controlli, e ciò varrebbe a confermare l'affermazione di G. Hertwig (1930), che il loro sviluppo è un poco ritardato. Di questi embrioni non furono però fatti preparati sufficienti a permettere un'affermazione sicura su questo punto.

Non ebbi mai a trovare le anomalie nella pigmentazione (albinis-

mo) segnalate dal Born e dal Hertwig, ma m'imbattei invece in un altro fenomeno degno di considerazione. Alcuni dei girini ibridi crebbero molto più degli altri e degli stessi controlli, tanto che, a circa 3



FIGG. 30–36.—Profili di embrioni, dell'età di 5 giorni, provenienti da uova di *Bufo vulgaris* (fec. eseguita il 15. V. 1931) disegnati dal vivo. Fig. 30. Controllo normalmente fecondato. Figg. 31–36, ibridi. (Ingr. 13 diam.)

settimane dalla fecondazione, in ogni vasca si trovavano alcuni individui di dimensioni superiori alla norma. A 5 settimane dalla fecondazione fu misurata la lunghezza totale di 87 girini, vivi, provenienti da tre vasche, che erano state sempre mantenute nelle stesse condizioni di temperatura e di alimentazione. I risultati sono rappresentati nella tabella II, e la curva fu espressa graficamente in una precedente nota (Montalenti, 1932).

TABELLA II

LUNGHEZZA TOTALE DI GIRINI IBRIDI DEL SECONDO INCROCIO, A 5 SETTIMANE DALLA FECONDAZIONE (FEC. DEL 13, IV. 1930)

Lunghezza in mm.	Numero degli Individui	Lunghezza in mm.	Numero degli Individui
9.5	1	16.5	.....
•	.....	17	3
11	1	17.5	1
11.5	1	18	1
12	6	18.5	2
12.5	2	19	.....
13	21	19.5	2
13.5	7	20	1
14	20	20.5	1
14.5	3	•	.....
15	8	•	.....
15.5	2	24	2
16	2	Totale	87

Come si vede la maggiore frequenza è fra i valori di 13 e 14 mm., la media fra tutte le misure è di 14.5. Oltre a un girino molto più piccolo del normale, che è un sopravvissuto dei girini inibiti di cui sopra ho riferito, se ne trovano alcuni che hanno dimensioni notevolmente superiori alla media, e alcuni relativamente giganteschi (da mm. 19.5 a 24). I controlli coetanei presentavano dimensioni oscillanti intorno a circa mm. 15, ma con una ampiezza di variazione incomparabilmente minore.

Sorse quindi il sospetto che si trattasse di individui poliploidi, e, non volendo sacrificarli, le code di un certo numero di ibridi di diverse dimensioni furono tagliate e i nuclei di diversi tessuti misurati. Non si potè però trovare la conferma della poliploidia, poichè non fu possibile riconoscere una differenza significativa fra i volumi dei

nuclei. Non rimane quindi, come spiegazione di questi individui giganti, che considerarli come casi di accelerazione di sviluppo.

Casi simili, come già dissi, furono incontrati dal Newman, che, in certi incroci fra specie diverse di *Fundulus*, sempre trovò alcuni individui che schiudevano precocemente e "were distinctly more active, grew faster and lived longer than the control" (Newman, 1917).

Una riprova del fatto che non si trattava di poliploidismo si ebbe alla metamorfosi: i girini giganti furono sempre più precoci, formarono precocemente gli arti e metamorfosarono prima degli altri. Questi rimasero sempre più piccoli (benchè la differenza tendesse poi a scomparire) ma le dimensioni dei piccoli rospetti metamorfosati non risultarono tanto diverse quanto quelle dei girini di 5 settimane. Inoltre tutti i girini giganti condussero a termine la metamorfosi, e ciò prova le loro migliori condizioni di vitalità, perchè fra gli altri si manifestò una mortalità non lieve, seppure non sensibilmente superiore a quella dei controlli.

In conclusione, i fenomeni dello sviluppo degli ibridi di questo incrocio richiedono di essere esaminati con maggior cura, ma si può fin d'ora affermare che si verifica una variabilità molto maggiore che non nella fecondazione normale del *B. vulgaris*, e che può fare il parallelo con quella già incontrata nell'incrocio reciproco. Si osservano qui poche gastrulazioni anomale, alcuni casi di sviluppo deficiente, che manifestano lievi inibizioni della morfogenesi, un gran numero di embrioni che si sviluppano normalmente, e un certo numero di girini che assumono proporzioni gigantesche. Questi conviene interpretarli come casi di accelerazione dello sviluppo, o di sviluppo migliore del normale, in attesa di conoscere meglio le cause che determinano il singolare fenomeno.

Il comportamento dei caratteri ereditarî paterni e materni formerà oggetto di altre comunicazioni; ricorderò qui soltanto che alcuni caratteri paterni (proprî del *B. viridis*) sono assai evidenti negli ibridi. Così la pigmentazione del corpo, se non è di tipo schiettamente paterno, come il Born aveva affermato, presenta tuttavia alcune macchie caratteristiche dei giovani rospi verdi, come quelle oculari, del braccio e dell'avambraccio, della coscia e della gamba. Ciò dimostra non solo l'avvenuta anfimissi, ma la possibilità dei geni paterni di determinare la formazione dei rispettivi caratteri.

VI. DISCUSSIONE

A. IL DIVERSO SUCCESSO DEGLI INCROCI

Molte ipotesi sono state avanzate per spiegare il diverso successo dei varî incroci di Anfibî, di Pesci e di Echinodermi, che sono stati i più completamente studiati, e in particolare, di alcuni incroci reciproci.

L'idea di una azione tossica che la cromatina paterna potrebbe esercitare sull'uovo e sull'embrione, sostenuta, fra gli altri, da Moenkhaus e J. Loeb, deve essere abbandonata, e lo è stata di fatto dagli autori successivi, perchè fa ricorso ad un'azione per nulla dimostrata, non spiega affatto il diverso comportamento degli incroci reciproci, e, in alcuni casi, è apertamente contraddetta dai fatti.

Il Newman (1908) e G. e P. Hertwig (1914) nelle loro ricerche sull' ibridazione eterospecifica di Teleostei, riconobbero che spesso gli ibridi che provengono da uova più voluminose e ricche di tuorlo si sviluppano meno bene di quelli che provengono da uova piccole, e muoiono senza avere esaurito le riserve vitelline. Avanzarono perciò l'ipotesi che una causa dell'insuccesso dell'ibridazione potesse dipendere dal fatto che "der Bastardembryo, der eine mittlere Grösse zwischen den Elternindividuen einzunehmen strebt, kann die für seinen Bedarf viel zu grosse Dottermenge der voluminösen Eier nicht bewältigen; die Herzaktion versagt und der Embryo stirbt ab" (G. e P. Hertwig, 1914). Non sempre però, nei pesci, questa interpretazione risulta applicabile, e nel caso dei rospi le condizioni sono opposte: è l'uovo più piccolo e meno ricco di tuorlo che si sviluppa imperfettamente, mentre quello più voluminoso dà origine a embrioni vitali.

G. Hertwig (1930) elaborò perciò un'altra interpretazione basata sull'ipotesi che il nucleo elabori un fermento specifico (*artspezifisch*) per la digestione del tuorlo. Nel nucleo ibrido perciò soltanto la parte di origine materna sarebbe attiva agli effetti della digestione del tuorlo. L'autore misurò il volume dei nuclei delle due specie parenti e trovò un rapporto eguale a circa 60 (*B. viridis*): 100 (*B. vulgaris*). Il volume delle cellule sta nella stessa proporzione, cioè il rapporto nucleo-plasmatico è eguale nelle due specie. Negli ibridi del secondo incrocio trovò volumi nucleari e cellulari intermedî fra quelli dei

parenti, e, in particolare, per i nuclei un valore prossimo a quello che può prevedersi teoricamente dalla precedente relazione, cioè 80. Ciò dimostra che deve essere avvenuta una regolazione del volume cellulare, per adattarlo a quello del nucleo ibrido.

Il Hertwig ritiene dimostrato che anche nell'ibrido reciproco si verifichino le stesse condizioni: regolazione del rapporto N/P e volume nucleare intermedio fra quelli delle specie parenti. Con un ragionamento che non è possibile seguire qui nei particolari, l'autore conclude che gli ibridi del primo incrocio posseggono, su pari volumi nucleare e plasmatico, minor quantità di sostanza nucleare della propria specie, che non gli ibridi del secondo incrocio. I primi avrebbero dunque una quantità di sostanza nucleare capace di secernere il fermento digerente il tuorlo minore di quella dei secondi (nel rapporto di circa 1.20:2) mentre ambedue hanno, proporzionalmente al volume totale del nucleo, la stessa quantità di tuorlo da utilizzare. Gli ibridi del primo incrocio si trovano perciò in condizioni più sfavorevoli di quelli del secondo, e ambedue, naturalmente, in condizioni di inferiorità rispetto agli embrioni normali.

L'ingegnosa argomentazione del Hertwig è seducente, e rappresenta un tentativo di interpretare in termini quantitativi questi fenomeni. Si basa però su due supposizioni: (1) che la sostanza nucleare secerna fermenti specifici per la digestione del tuorlo; (2) che anche negli ibridi del secondo incrocio avvenga la regolazione del rapporto N/P e conduca alla stessa relazione numerica. La prima ipotesi è difficile a verificare sperimentalmente e, benchè l'autore consideri i fenomeni su descritti come una sua prova indiretta, è finora indimostrata. La seconda, benchè non improbabile, non è corroborata dai dati della misura diretta. Questa teoria inoltre non sembra, allo stato attuale delle conoscenze, passibile di applicazione generale, e non getta alcuna luce sui fenomeni che si osservano nella embriogenesi dei due ibridi.

Mi pare che sia possibile un'interpretazione quantitativa dei fenomeni descritti, che non richiede l'intervento di ipotetiche sostanze specifiche, la cui presenza ed azione sono del tutto oscure. È quella che tien conto delle velocità di sviluppo delle due specie, e che già avanzai in una precedente nota (Montalenti, 1932). Non è nuova, ma può forse essere espressa in forma più precisa di quanto non sia

stato fatto finora, soprattutto se si tien conto delle recenti ricerche sulla fisiologia e la dinamica della divisione cellulare.

Già il Newman aveva diligentemente misurato le velocità di sviluppo delle due specie parenti, e osservato altre caratteristiche fisiologiche, che probabilmente vi sono correlate. Aveva anche segnalato la relazione che esiste fra le velocità di sviluppo degli ibridi e il risultato della ontogenesi, ed era giunto alle conclusioni già riferite (pag. 355). Evidentemente egli era incline ad una interpretazione che invocasse appunto la differenza fra le velocità di sviluppo caratteristiche dei due gameti che si accoppiano, come causa del diverso successo della ontogenesi; ma alcune eccezioni gli impedirono di generalizzare questa conclusione.

Il problema può però essere posto in termini alquanto diversi. Parlando di "velocità di sviluppo" e riferendosi essenzialmente alla velocità di segmentazione dell'uovo, che può esserne considerata come un indice, non si può trascurare il rapporto N/P. È ben nota l'importanza del volume del citoplasma rispetto a quello del nucleo per la velocità del ritmo di divisione delle cellule. A parità di altre condizioni esterne ed interne, e a parità di volume nucleare, si divide più presto un uovo con scarso tuorlo, che non uno più ricco di deutoplasma. Perciò non è lecito fare un paragone in termini assoluti delle velocità di segmentazione di due uova, con una semplice comparazione degli stadî in relazione al tempo e mantenendo fisse le altre variabili (temperatura, ecc.). Si deve bensì tener conto del rapporto N/P iniziale, per poter ridurre i due ritmi alla stessa scala, apportando quelle correzioni che la suddetta relazione può suggerire. Nel caso in cui le cellule abbiano lo stesso rapporto N/P, la condizione è invece senz'altro soddisfatta.

Il ritmo di segmentazione, quando sia correttamente interpretato, può forse essere considerato come un indice rappresentativo di caratteristiche fisiologiche proprie delle singole specie. Non è certo il risultato di una semplice correlazione di ordine meccanico, perchè, in questo caso, tutte le cellule con egual rapporto carioplasmatico dovrebbero dividersi con ritmo eguale, e sappiamo che ciò non è vero. Basta pensare poi al complesso ciclo di fenomeni fisiologici connesso con il processo della divisione cellulare, per convincersi che il ritmo cinetico deve essere la risultante di proprietà caratteristiche dei

plasmi. Mutamenti della permeabilità, delle relazioni fra le fasi dei colloidi, e di altre proprietà fisiche e chimiche del plasma, sono stati studiati in molti casi e le loro relazioni con le varie tappe del processo morfologico della divisione cellulare stabilite. Essenzialmente simili in tutte le cellule, queste fasi del ritmo fisiologico presentano però caratteristiche proprie per ogni specie, per ogni sorta di cellule, e devono essere quindi un'espressione di proprietà specifiche dei varî plasmi.

Comunque è certo che il ritmo cinetico ha un'importanza fondamentale nello sviluppo dell'uovo e che le sue variazioni si ripercuotono profondamente sul processo dell'ontogenesi. Fin dalle ricerche di Herbst e in tutti i lavori sulla partenogenesi sperimentale e la fisiologia della fecondazione, le conseguenze dei ritmi anormali o disarmonici si sono imposte all'attenzione degli sperimentatori. Ma è merito soprattutto delle ricerche del Bataillon l'aver messo in evidenza, con un'accurata analisi dei cicli cinetici normali e anormali delle uova, l'importanza della regolazione dei ritmi dei due pronuclei per il successo dell'evoluzione.

Se si tien conto di questi fatti e se si considera l'alterazione della velocità di sviluppo, evidente soprattutto nei casi in cui si manifestano le inibizioni, appare molto probabile che vi sia un legame fra l'asincronia dei ritmi cinetici delle due specie, che deve essere causa di un notevole squilibrio delle mitosi, e il risultato della ontogenesi.

Già il Baltzer (1910) aveva preso in considerazione questo fatto, come base per una possibile interpretazione dell'epurazione cromatica di alcuni dei suoi incroci fra Echinodermi, e l'aveva scartato, dopo lunga discussione. Egli si riferiva però semplicemente al ritmo di segmentazione, senza considerare il volume delle uova e il rapporto N/P. Benchè alcune delle sue obiezioni siano tutt'altro che trascurabili, è pur sempre possibile che la sua critica possa essere riveduta.

Il significato dell'asincronia non sfuggì al Tchou (1931) che, nel già citato lavoro, così si esprime: "Mais quelle est la condition de ces éliminations totales ou partielles? Pourrait-on facilement substituer à la notion d'incompatibilité entre plasma maternel et spermie étrangère, ou chromosomes étrangers, celle d'une dysharmonie dans leur mouvements, dans leur résolution en chromosomes?" E cita il caso

dei due incroci reciproci fra *Hyla* e *Bufo*, che portano tutti e due a embriogenesi incompleta, a molti casi di eliminazione della cromatina paterna e ad alcuni falsi ibridi aploidi. L'uovo di *Bufo*, secondo il Tchou, è più lento di quello di *Hyla*, nel quale il primo solco compare un'ora e mezza prima.

Anche Lillie e Just (1924) sono poco propensi ad ammettere che fattori specifici intervengano dopo l'entrata dello spermio e l'avvenuta reazione corticale dell'uovo. Esaminando i casi descritti di ibridazione fra Echinodermi, e constatando come, quasi sempre, per ottenere la fecondazione, si sia dovuto ricorrere al trattamento delle uova o degli spermî con qualche reattivo, giungono a questa conclusione: "It is not necessary, therefore, to assume a specific factor as responsible for chromatine elimination in hybrid fertilization. The elimination may be merely the expression of disharmony between the egg nucleus and sperm nucleus, the tempo of their movements being altered because of the treatment on the egg plasma before or after the fertilization." Se il ritmo cinetico è normalmente diverso nelle specie parenti, si giunge alla stessa conclusione anche dove non vi sia l'intervento sperimentale segnalato dagli autori americani.

Nei casi qui presi in esame, la diversità del ritmo cinetico è fuori di dubbio: la segmentazione del *B. vulgaris* è, come si è detto (pag. 359), alquanto più lenta, e poichè il rapporto N/P è uguale nelle due specie, è ragionevole ritenere che lo sia anche nell'uovo. La velocità di segmentazione è quindi un buon indice della asincronia dei ritmi cinetici, la quale è relativamente di poca entità.

Con l'introduzione di uno spermio a ritmo lento (*B. vulgaris*) nell'uovo a più rapido sviluppo (*B. viridis*) l'ontogenesi è notevolmente alterata. Nel caso reciproco invece, con l'introduzione di uno spermio a ritmo più rapido in un uovo più lento, si ottiene una buona percentuale di embrioni normali e vitali.

Anche negli incroci *Bufo* × *Hyla*, eseguiti da Bataillon e Tchou, sembra che si verifichino condizioni simili: la combinazione *Hyla* ♀ × *Bufo* ♂ dà luogo a stereoblastule e a "gastrulations abortives," oltre a un certo numero di larve aploidi: la combinazione reciproca invece dà origine a poche stereoblastule, a gastrule anormali, che producono spina bifida, asintassia del blastoporo, ecc., e a un certo

numero di girini che muoiono a varî stadî nel corso dello sviluppo, alcuni dei quali si rivelano aploidi. Il risultato generale sembra dunque migliore in questo incrocio. Valgono per questo caso, come si è detto, le stesse relazioni fra i ritmi di sviluppo che si osservano nei rospi, ma l'asincronia, a quanto risulta dai dati del Tchou, sembra più marcata.

Anche nei casi descritti dal Newman per i Teleostei e gli Echinodermi si verificano per lo più condizioni analoghe, come si è già detto, e le eccezioni andrebbero riesaminate soprattutto per quanto concerne il rapporto carioplasmatico.

Se è vera dunque l'interpretazione proposta, che cioè le relazioni fra i ritmi cinetici delle due specie parenti siano responsabili del diverso risultato dell'embriogenesi, si deve concludere che è meno dannosa l'introduzione nell'uovo di uno spermio a più rapido ritmo di sviluppo, che non di uno a ritmo più lento. E tale conclusione si accorda pienamente con i fatti osservati, sia nella maggior parte dei casi di ibridazione, che in tutti i casi di alterazione sperimentale del ritmo di sviluppo: sempre ad un rallentamento notevole del ritmo evolutivo corrisponde l'insorgenza di alterazioni dello sviluppo embrionale.

Rimane a vedere se e quando è veramente constatabile, nel caso dell'ibridazione, un'alterazione del ritmo di sviluppo. In generale gli autori sono concordi nell'affermare che la segmentazione avviene con il ritmo normale della specie madre. Tuttavia le misure eseguite non sono state in generale molto precise, e la questione dovrebbe essere ripresa in esame. Il Newman (1910) infatti, con accurate misure e con la statistica dei risultati, potè dimostrare che, nelle uova di *Fundulus majalis* fecondate con spermî di *F. heteroclitus* si osserva, in media, una accelerazione del processo di segmentazione, accelerazione che si mantiene in seguito, nel corso dello sviluppo. In altri casi accenna ad un rallentamento dello sviluppo, ma non si pronunzia per quel che riguarda il ritmo di segmentazione.

Nelle presenti ricerche questo particolare non fu oggetto delle precise misure che avrebbe meritato, e nulla ci è permesso affermare su quanto riguarda il ritmo di segmentazione. Nel primo incrocio, un rallentamento evidente e notevole si rende manifesto a cominciare dalla gastrulazione. Nel secondo si verificano alcuni casi di sviluppo

migliore del normale, che si sono considerati come dovuti ad accelerazione.

La questione deve essere riveduta prima di poter formulare affermazioni sicure, ma, dal complesso dei risultati, risulta l'analogia con quelli descritti dal Newman per i Pesci, e la sua conclusione: "introducendo in un uovo uno spermio estraneo, se ne accelera o rallenta la velocità di sviluppo" pare pienamente applicabile ai casi qui esaminati. Aggiungeremo che a priori non sembra improbabile (1) che l'azione modificatrice della velocità di sviluppo si manifesti soprattutto allorchè le cellule, finita la segmentazione, hanno acquistato il rapporto N/P normale e sia perciò più sensibile dopo che durante la segmentazione; (2) che l'introduzione di un fattore di ritardo nello sviluppo abbia effetti più vistosi e dannosi che non un fattore di accelerazione, soprattutto quando la discordanza di fase dei ritmi non sia molto notevole.

L'interpretazione su esposta presenta notevoli vantaggi su ogni altra, innanzi tutto perchè è a base principalmente quantitativa, e poi perchè se ne può prevedere l'applicazione a tutti i casi: eliminazione precoce e totale o tardiva e parziale della cromatina, e anfimissi con disturbo più o meno grave della organogenesi, cioè a ogni caso in cui avvenga la reazione corticale e la penetrazione di uno spermio. Nei casi di mancata reazione il problema è diverso e occorre probabilmente fare appello a sostanze specifiche: il Lillie, che ha analizzato profondamente i processi relativi alla reazione corticale e alla penetrazione dello spermio, è del parere che in questi soltanto risieda il carattere specifico della reazione.

Terzo e non ultimo vantaggio è la possibilità che ci è offerta di trovare una relazione fra la causa dell'insorgenza delle inibizioni morfogenetiche descritte e la causa di quelle che si ottengono con altri mezzi sperimentali, come verrà discusso nella prossima sezione. È poi da tener presente, che, per quanto sopra si è accennato, lo squilibrio dei ritmi cinetici, può considerarsi come una espressione di specificità in termini quantitativi.

Alcune difficoltà, non tutte lievi, si possono prevedere, per l'applicazione della interpretazione suddetta, come i casi di eliminazione parziale di determinati gruppi di cromosomi, fin dalla prima mitosi, descritti dal Baltzer, o il fatto che è sempre la cromatina paterna

quella che viene eliminata. Comunque, per tutti i vantaggi che presenta, ritengo che tale interpretazione sia meritevole di essere presa in seria considerazione, e la sua validità generale provata al paragone dell'esperimento.

B. L'INIBIZIONE DIFFERENZIALE

1. *Cause dell'inibizione.*—Scartata la possibilità di un'azione tossica della cromatina estranea e ammessa l'ipotesi che la causa primaria dei disturbi dell'ontogenesi sia lo squilibrio dei ritmi cinetici, occorre ricercare in qual modo ci si possa render conto delle inibizioni osservate, e se sia possibile trovare un punto di contatto con l'azione degli altri fattori teratogeni fin qui sperimentati.

Innanzi tutto conviene ricordare che, in seguito a quanto hanno dimostrato le ricerche del Tchou, è probabile che si possano verificare, soprattutto nel primo incrocio, casi diversi di eliminazione tardiva e parziale della cromatina paterna, similmente a quanto avviene nelle combinazioni *Hyla* × *Bufo*. Questi possono spiegare la formazione di stereoblastule e alcune delle anomalie più gravi della gastrulazione, specialmente quelle che conducono alla formazione di embrioni asimmetrici. È una possibilità, che per venire dimostrata richiede tuttavia l'esame citologico. Ma sempre rimangono tutti gli altri casi in cui la diploidia è certa e che presentano le malformazioni caratteristiche descritte.

Come già si è detto, effetto comune a tutti gli agenti inibitori è, per concorde affermazione degli autori, un'alterazione (per lo più una diminuzione) della velocità di sviluppo, cioè a dire, in ultima analisi, un'alterazione (rallentamento) dei ritmi divisorî o, se si vuole, dei processi fisiologici del plasma connessi con le mitosi. Se questa sia la causa prima delle inibizioni, o si tratti di semplice coincidenza, è vano, per ora, ricercare. Certo è che la relazione esiste, e, per quanto io sappia, non conosce eccezioni.

Già abbiamo accennato ai complessi fenomeni ritmici che avvengono nel plasma durante le divisioni; i più imponenti sono i cambiamenti di viscosità, cioè a dire le relazioni fra le fasi dei colloidi plasmatici. Questi sono il risultato di un equilibrio nelle relazioni nucleo-plasmatiche, come numerose ricerche, e in particolare (per l'uovo degli Anfibî) quelle recenti del Dalcq (1928, 1929, 1930, 1931) e collaboratori hanno dimostrato. Questo autore ha messo in evi-

denza soprattutto due fatti di molto interesse; (1) l'importanza del succo nucleare nella formazione e nel ritmo degli aster su cui si centrano le mitosi; (2) la funzione dinamica dei cromosomi nella plasmodieresi. Quando, con il radio o la tripaflavina o altri mezzi, il Dalcq altera la compagine di uno dei gameti, si producono nelle mitosi e nelle plasmodieresi varî disturbi dipendenti dalle modificazioni di uno o di ambedue questi costituenti nucleari.

L'interpretazione basata sull'asincronia dei ritmi evolutivi può render ragione dei fenomeni della inibizione della organogenesi facendo appello a un'alterazione delle relazioni nucleo-plasmatiche, soprattutto per quanto si riferisce ai fenomeni di gelificazione e ai cambiamenti di viscosità del plasma che si manifestano in relazione ai processi cinetici. Lo sfasamento dei ritmi, se molto accentuato, può spiegare i casi di eliminazione totale alla prima mitosi, per l'asincronia della risoluzione in cromosomi dei due pronuclei; se meno grave può produrre squilibrî tali nelle relazioni nucleo-plasmatiche di ogni cellula, da intralciarne il normale processo di sviluppo.

All'alterazione degli equilibrî nucleo-plasmatici e all'importanza delle condizioni di viscosità del plasma in relazione alle malformazioni ottenute con la centrifugazione, si riferiscono Pasquini e Reverberi (1929) nel già citato lavoro, per spiegare sia la suscettibilità diversa nei varî momenti della vita embrionale alle aggressioni sperimentali, sia la genesi delle inibizioni. Dopo un'ampia discussione sull'argomento essi emettono l'ipotesi che l'effetto primario di ogni azione inibitrice, sia da ricercarsi in un'alterazione degli equilibrî fra i colloidi del nucleo e quelli del plasma, ipotesi che è stata anche recentemente accettata dal Chiarugi (1932).

Questa interpretazione, a cui gli autori sono giunti per via totalmente diversa da quella per cui conduce l'esame delle inibizioni da ibridazione, mi sembra pienamente accettabile. È appoggiata da molti fatti ben conosciuti circa l'effetto che varie sostanze (sali, anestetici, ecc.) e azioni fisiche (irradiazioni, temperatura, ecc.) esercitano sulla viscosità, permeabilità e altre proprietà fisiche e chimiche del plasma, e non urta contro nessuna delle obiezioni che si parano dinanzi alle altre, che fanno appello ad azioni specifiche. Rappresenta un tentativo d'interpretazione unitaria dei fenomeni di inibizione, ed è atta a dar ragione anche delle inibizioni da ibridazione e

di quelle derivate da azioni esercitate su uno solo dei gameti. Nell'un caso per condizioni naturali, nell'altro in seguito a intervento sperimentale, il ritmo di uno dei due pronuclei è rallentato rispetto all'altro. Anche qui, come nei casi di azioni dirette sullo zigote o sull'embrione, il risultato è molto probabilmente un'alterazione dell'equilibrio fisico-chimico dei processi ritmici dei colloidi cellulari, che si manifesta immediatamente con l'alterato processo evolutivo.

Che l'azione dei sali, delle irradiazioni, della temperatura, ecc. possa determinare un'alterazione del ritmo di sviluppo, è ben noto. Perciò quei casi in cui l'azione sperimentale è diretta su uno solo dei gameti sono assimilabili, in certo modo, ai casi di ibridazione fra due specie a ritmi asincroni.

Ricorderemo che il parallelismo fra l'effetto, ad es., dell'irradiazione degli spermî e i diversi casi che si incontrano nelle ibridazioni eterospecifiche degli Anfibî è perfetto: con irradiazioni di intensità sempre crescente si ottengono tutti i casi descritti nella ibridazione. Da inibizioni relativamente lievi a profondi disturbi dell'organogenesi, ad anomalie più o meno gravi della gastrulazione, fino a totale assenza di gastrulazione, e finalmente a epurazione cromatica e a sviluppo partenogenetico ("effetto paradossale" degli Hertwig).

Inoltre l'analogia fra le inibizioni qui descritte e quelle ottenute con l'irradiazione è tutt'altro che superficiale, e su di essa ho già avuto occasione di richiamare l'attenzione. Non soltanto le alterazioni della morfogenesi, ma i fenomeni istologici (emissione di cellule, formazione e deposito di pigmento, ecc.) determinati dalla *Radiumkrankheit* trovano perfetto riscontro, fin nei più minuti particolari, nelle anomalie che si verificano nelle inibizioni da ibridazione.

Ricorderemo ancora il fatto messo in luce da Dalcq e Simon (1931), che l'uovo di rana sembra meno suscettibile dello spermio all'effetto dell'irradiazione. Ciò potrebbe forse essere interpretato nel senso che un ritmo disarmonico in cui lo spermio è in anticipo sull'uovo ha effetto meno grave, per il normale corso dell'ontogenesi, che non un ritmo disarmonico in cui lo spermio è in ritardo, come nel caso dei nostri incroci.

È opportuno ripetere che tutte queste considerazioni sul tempo dei ritmi non escludono affatto l'idea della specificità e neanche di alterazioni chimiche o fisiche particolari, indotte dagli agenti terato-

geni: l'alterazione del ritmo di sviluppo non sarebbe altro che l'espressione tangibile di questi fatti.

2. *La suscettibilità differenziale.*—Qual sia la causa dell'inibizione differenziale, che fa sì che alcuni territorî dell'embrione siano più suscettibili di altri all'azione perturbatrice, è una domanda che si sono fatti tutti coloro che hanno studiato la teratogenesi sperimentale, e alla quale sono state date varie risposte. L'ipotesi di un'azione specifica di certe sostanze su determinati tessuti o territorî embrionali, si è dovuta scartare, come già si disse. Del resto ogni concezione che involva l'idea di una specificità non meglio dimostrata e non definita in termini chimici o fisico-chimici, è destinata a lasciare insoddisfatti.

Il merito di avere posto questo problema su terreno più fermo e di averlo sottoposto ad una analisi quantitativa, spetta al Child e alla sua scuola (bibliografia in Child, 1924, 1928). Con una serie di ricerche, condotte dapprincipio specialmente sugli invertebrati, questo autore giunse alla conclusione che le parti del corpo, che presentano un metabolismo più elevato, risentono più prontamente e più profondamente l'azione di certi agenti tossici, a dosi opportune. La suscettibilità differenziale (a cui il Child riconduce diversi fenomeni: morte, inibizione, acclimazione, restituzione differenziale) sarebbe dunque l'espressione di differenze nel livello di metabolismo delle varie parti del corpo.

Con varî metodi, che qui non occorre riassumere, il Child ha poi dimostrato che esistono negli organismi certe gradazioni delle intensità di metabolismo, o "gradienti metabolici", o più generalmente fisiologici, disposti lungo certi assi. Il gradiente primario e principale, negli organismi che presentano una polarità, sarebbe il gradiente anteroposteriore, al quale, durante il corso dello sviluppo, possono venire ad aggiungersene altri, secondarî, diversamente orientati. Sui numerosi dati sperimentali raccolti da lui e dai suoi collaboratori, il Child ha poi elaborato la "teoria dei gradienti", che assegna a questi un'importanza fondamentale nella direzione dello sviluppo organico e nei processi del differenziamento. Sarebbe qui fuor di luogo entrare nei particolari di questa teoria, e ne discuteremo soltanto l'importanza per la spiegazione delle inibizioni differenziali.

Nel discutere la teoria dei gradienti, in quanto applicabile al nos-

tro caso, è opportuno, io credo, distinguere due momenti, che, sebbene logicamente e intimamente connessi, possono, fino a un certo punto, considerarsi indipendenti: (1) la relazione fra livello di metabolismo e suscettibilità differenziale; (2) la presenza di gradienti fisiologici (di cui il fenomeno suddetto è, secondo il Child, una delle prove) e la loro possibile funzione direttiva nello sviluppo organico.

Che le regioni a più elevato metabolismo sieno quelle che risentono maggiormente l'azione perturbatrice, è, per quanto riguarda i Vertebrati, un'ipotesi che ha molte prove in suo favore, e anche a priori sembra molto verisimile. La conferma sperimentale è irta di difficoltà, dovute soprattutto all'imperfezione della tecnica microfisiologica, ma, allo stato attuale delle ricerche non mi pare che i dati raccolti dal Parker (1929) e dallo Shearer (1930) che sembrano parlare contro la teoria dei gradienti, possano considerarsi decisivi, di fronte al gran numero di prove favorevoli.

Recentemente il Ranzi (1927, 1929*b*, 1931, 1932) sottopose la teoria del Child a una acuta critica, particolarmente per quanto riguarda la sua applicazione ai fenomeni dello sviluppo. Egli si basa soprattutto su argomenti tratti dalla embriologia, e in particolare dalle proprie ricerche sulla suscettibilità differenziale nei Cefalopodi. Per quello che si riferisce alla teratogenesi, il Ranzi intende sostituire al principio enunciato dal Child il seguente: "gli organi sono tanto più facilmente e profondamente inibiti, quanto più complessa è la maniera della loro formazione" (Ranzi, 1928, 1931). Evidentemente ciò non esclude, anzi conferma la relazione fra livello di metabolismo e grado di suscettibilità stabilita dal Child. Poco oltre, infatti, lo stesso autore così si esprime: "vediamo infatti che i processi disintegrativi, determinati dall'azione degli agenti tossici, si iniziano sempre dove è, nel momento dell'azione, in corso un processo organogenetico e questo è logico, perchè le cellule che si spostano hanno, come vedremo, un metabolismo più attivo delle cellule che si muovono meno, quindi soccombono più facilmente ad un'azione tossica" (Ranzi, 1931, pag. 22). Quindi tutti i "processi a cui corrispondono più notevoli spostamenti di cellule" sono, per ciò stesso, caratterizzati da un più elevato livello di metabolismo.

La critica del Parker (1929), il quale afferma che la sostanza nervosa non ha un più elevato metabolismo (tant'è vero, egli dice, che per

scaldarci quando siamo intirizziti, esercitiamo i muscoli, anzichè pensare fortemente, *loc. cit.*, pag. 424) non corrisponde a quanto è generalmente riconosciuto dai fisiologi: che la sostanza cerebrale ha un elevatissimo quoziente respiratorio e metabolismo più intenso che non i muscoli e il fegato (cfr. Mathews, 1928). Una prova indiretta ne è data anche dalla copiosa e fine irrorazione sanguigna del cervello.

A questa affermazione, dunque, di una relazione fra il livello di metabolismo e grado di suscettibilità, dimostrata, in molti casi, da numerose ricerche, mi pare che, allo stato attuale delle conoscenze, non possano essere opposti serî argomenti. È una interpretazione che, per quanto riguarda le inibizioni differenziali nel corso dell'embriogenesi dei Vertebrati, è (conviene ricordarlo) essenzialmente teorica, ma non saprebbe essere sostituita per ora da una migliore. È certamente l'unica che possa gettare luce, oltre che sui fenomeni della inibizione della organogenesi, sulla diversa suscettibilità dei varî tessuti. Disponendo i tessuti in ordine di crescente suscettibilità (così come ci è rivelata dalla gravità dell'alterazione a cui soggiacciono) si ottiene infatti la stessa serie che si avrebbe ponendoli in ordine di crescente attività metabolica, complessità dei processi di organizzazione chimica, e perciò, come vuole il Parker, delicatezza degli equilibrî di ogni singola cellula. Ciò in base a quanto ci consentono le scarse conoscenze attuali sul metabolismo dei singoli tessuti.

Rimane il secondo punto, che è il nodo della costruzione teorica del Child, se cioè esistano i gradienti fisiologici e quale possa essere la loro funzione nell'embriogenesi. Varie critiche sono state mosse alle affermazioni dell'autore americano: fra le più recenti e documentate sono quelle del Parker e del Ranzi, già citate. Partendo da criterî eminentemente fisiologici l'uno, da considerazioni embriologiche l'altro, i due autori si accordano nel sostenere che le differenze di metabolismo non sono da considerarsi come fenomeni formativi o direttivi dello sviluppo e del differenziamento. Il Ranzi—la cui critica è più ampiamente documentata e solleva alcune gravi obiezioni alla teoria dei gradienti—fa inoltre osservare che "le zone a più elevato metabolismo sono circoscritte e le zone vicine non presentano un metabolismo gradatamente decrescente man mano che ci si allontana dalla zona a più elevato metabolismo, ma solo un metabolismo di-

verso"; che i centri a più elevato metabolismo hanno durata transitoria e non hanno la posizione prevedibile in base alla teoria. Entra cioè in merito alla questione dell'esistenza dei gradienti, e la nega, dopo un accurato esame dei fenomeni dell'ontogenesi.

Come si vede la disputa tocca un tasto molto delicato e difficile della ricerca scientifica: la relazione di causa ad effetto, e si può, in ultima analisi, riassumere nella seguente domanda: sono i gradienti, o comunque le differenze di metabolismo causa o effetto del differenziamento morfologico?

Senza entrare in merito a sì delicata questione, che esula dallo scopo delle presenti ricerche, mi limiterò ad associarmi all'opinione già professata da numerosi ricercatori, che i fenomeni che si osservano nell'inibizione differenziale parlano a favore di una gradazione fisiologica lungo il neurasse, in senso cefalo-caudale, com'è ammesso dalla teoria dei gradienti. Abbiamo visto infatti che le malformazioni ottenute interessano specialmente la parte craniale del sistema nervoso, e sono tanto più limitate alla regione rostrale quanto meno intensa è l'inibizione.

3. *Meccanismo d'insorgenza delle alterazioni.*—Il meccanismo d'insorgenza delle alterazioni derivanti dall'inibito processo di gastrulazione è stato recentemente studiato dal Goerttler (1926) in base alle nozioni sulla topografia delle localizzazioni embrionali nella blastula degli Anfibî (Vogt) e sui movimenti del materiale durante il processo di gastrulazione (Mangold, Vogt, Goerttler). L'autore ha analizzato tutti i casi che si ottengono con lesioni del labbro dorsale del blastoporo e trattando gli embrioni di Urodeli con soluzioni saline (exogastrule, embrioni anulari, varî gradi di spina bifida, asintassia del blastoporo senza bipartizione degli organi assili). Giunse alla conclusione che la genesi di queste malformazioni sia determinata non da un impedimento dell'invaginazione, la quale avviene come di norma, ma piuttosto da inibizione dei movimenti di massa dei materiali degli abbozzi midollare e cordo-mesodermico, che devono venire a confluire cranialmente al blastoporo.

La disposizione degli abbozzi presunti negli Anuri non è essenzialmente differente da quella degli Urodeli (Vogt, 1929) e probabilmente i fenomeni che si verificano nei nostri casi sono simili. Il rallentamento del movimento verso il polo vegetativo del labbro dor-

sale del blastoporo, che è la zona di più attiva invaginazione, parla anche a favore di questa interpretazione; qui infatti vengono tosto a mancare i materiali d'invaginazione, a somiglianza di quanto si verifica dopo l'asportazione di una parte del materiale craniale al labbro dorsale.

Le malformazioni ottenute in seguito ad anomalie della gastrulazione, che non sono in alcun modo specifiche per le inibizioni da ibridazione, sarebbero perciò derivate da impedimento dei movimenti di massa dei blastomeri dei varî abbozzi e non da rallentamento dell'invaginazione o da un cambiamento di determinazione degli abbozzi presunti. Questi si differenziano come nell'embriogenesi normale, soltanto la loro posizione e le loro reciproche relazioni definitive non vengono raggiunte.

Nei casi di asintassia del blastoporo non accompagnata da spina bifida, i processi formativi, secondo il Goerttler, dovrebbero essere inibiti meno intensamente nella regione dorsale che non nelle laterali. In queste ultime perciò non avverrebbe il differenziamento del materiale midollare.

Le anomalie asimmetriche, molto frequenti e spesso estreme, nei nostri casi, non hanno ancora trovato una spiegazione soddisfacente. Si può pensare qui a eliminazione parziale della cromatina, o a sua irregolare distribuzione, in stadî più o meno precoci, ma occorre la verifica citologica, che, dato il carattere del materiale, non è facile.

I casi di anencefalia e emiencefalia, come si disse, sono derivati dalla mancata chiusura della parte anteriore delle pieghe midollari. Se queste rimangono aperte per tutta la loro lunghezza, si formano embrioni anidiani.

Anche le alterazioni dello sviluppo che si verificano quando la gastrulazione e la chiusura delle pieghe midollari sono avvenute normalmente, sono determinate dalla inibizione dello sviluppo e incompleto differenziamento di parti, anzichè da diverso destino o da spostamenti dei materiali dei presunti abbozzi. Si possono distinguere, nei nostri casi, anomalie primarie, risultato diretto della inibizione, e anomalie secondarie, di organi la cui inibizione è conseguenza del mancato o insufficiente sviluppo di altre parti.

Fra le inibizioni primarie è certamente quella che colpisce il sistema nervoso e si manifesta con particolare gravità nell'encefalo. L'i-

nibizione dell'encefalo può essere totale, con formazione di cervello interamente massiccio, o limitata alla parte più rostrale. In questi casi la parte precordale dell'encefalo è la più gravemente colpita, ma anche nei tratti successivi si possono trovare considerevoli anomalie, e obliterazione delle cavità. Tutto il neurasse, anche nella parte del tronco e caudale, risente gli effetti dell'inibizione.

In conseguenza dell'inibizione dell'encefalo e dell'insufficiente sviluppo delle sue cavità, può verificarsi la mancanza di cristallino (bilaterale in tutti i casi osservati) la quale dipende evidentemente dal mancato avvicinamento degli abbozzi oculari all'ectoderma lentogeno. Anche le inibizioni della regione rostrale, nella sua parte non nervosa, sono da interpretarsi come risultato secondario del difettoso sviluppo dell'encefalo: il grado di alterazione di queste parti è infatti strettamente connesso al grado di sviluppo raggiunto dal cervello. Entrano qui in giuoco quella funzione di sostegno e quelle correlazioni di ordine meccanico messe in luce dal Giardina (1914), e poi dal Cotronei (1915, 1919, 1922*a*) con lo studio dei fenomeni d'inibizione delle larve a litio. Il cervello precordale esercita, secondo quest'autore, un'azione di sostegno necessaria per il normale sviluppo degli organi circostanti (vestibolo orale, fossette olfattorie, lente, vescicole ottiche); quando questa viene a mancare, tali organi non trovano le condizioni di spazio necessarie al loro sviluppo e risultano perciò ridotti o assenti. Così si possono intendere l'inibizione del vestibolo orale, con la totale scomparsa delle labbra o la loro fusione in un tubercolo mediano, o, nei casi meno gravi, il mancato o insufficiente sviluppo delle cartilagini rostrali, ecc.

Anche l'atrofia delle branchie deve essere di carattere secondario e dipendere dall'inibizione che colpisce il cuore. Alcuni embrioni non raggiungono lo stadio in cui normalmente dovrebbero svilupparsi le branchie, in altri invece questi organi si formano, ma non possono raggiungere le dimensioni normali per l'insufficiente pressione sanguigna. L'importanza di questo fattore per il normale sviluppo delle branchie è stata infatti riconosciuta da lungo tempo da tutti gli autori che hanno studiato la formazione di questi organi (cfr. Ekman, 1913; Della Valle, 1914; Marchetti, 1914*b*; Harrison, 1921).

La curiosa curvatura del corpo, che s'incontra anche, sebbene non così pronunziata e costante, in embrioni irradiati, partenogenetici

e in ibridi fra altre specie, è dovuta evidentemente a insufficienza di sviluppo degli organi dorsali del tronco in confronto a quelli ventrali. È il risultato della inibizione, che sembra manifestarsi non tanto per il numero minore del normale di segmenti mesodermici, quanto per l'insufficiente sviluppo e differenziamento di questi. Mancano però a questo proposito conteggi esatti, e non fu ricercato se il numero dei somiti sia differente nei girini che presentano code di lunghezza diversa. Così pure è insoluta, nei nostri casi, la questione se e quando le connessioni nervose abbiano importanza per il differenziamento dei fasci muscolari. Ricorderemo, a questo proposito, che una delle ragioni per cui gli ibridi del primo incrocio manifestano una così scarsa mobilità, va cercata, più che nell'insufficienza delle connessioni nervose, nella debilità dei fasci muscolari.

È interessante notare, infine, che sembra esservi una correlazione reciproca fra il grado di sviluppo della corda dorsale e quello del sistema nervoso: nelle larve che procedono alquanto nel differenziamento, si rileva che quanto più è inibito il neurasse, tanto più la corda è ipertrofica.

Le inibizioni dell'encefalo sono di grado diverso, ma essenzialmente simili per quanto concerne le relazioni fra le singole parti: tuttavia si possono trovare alcune variazioni nei varî individui, come diverso spessore delle pareti, obliterazione delle cavità che in altri embrioni sono invece enormemente sviluppate, ecc. Questi fatti suggeriscono un diverso comportamento delle parti dell'encefalo nei varî embrioni, e da una loro analisi accurata su un gran numero di casi, si potrebbe trarre forse qualche conclusione circa le relazioni fra le diverse parti del cervello e il loro modo di reazione ai processi inibitorî.

Anche negli embrioni che hanno gastrulato normalmente, rimane sempre il problema dell'origine dell'asimmetria, talvolta così notevole e di frequente comparsa in tutti gli esperimenti eseguiti.

C. LA SERIE DI VARIABILITÀ DEI RISULTATI

In tutti gli esperimenti di ambedue gli incroci, si verifica costantemente una variabilità che è di gran lunga superiore a quella che si constata nelle fecondazioni normali. I limiti estremi della serie sono rappresentati, in un caso, da divisioni anormali e stereoblastule, e

individui pressochè normali, nell'altro da gastrulazioni anomale o inibizioni precoci, e sviluppi migliori del normale. Questi stessi fenomeni avevano già attirato l'attenzione del Newman nei suoi ibridi di Teleostei, e la loro costanza, e il ripetersi in due gruppi diversi consigliano di prenderli in considerazione.

Anche nella teratogenesi ottenuta con altri mezzi si verifica una notevole variabilità nel grado delle malformazioni ottenute, specialmente quando si opera con dosi intermedie fra le quasi innocue e le letali; in genere però la variabilità non sembra così ampia, nè così costante. Recentemente Dalcq e Simon (1931) accennano alla variabilità osservata negli esperimenti di irradiazione, e ricordano come il Vintemberger abbia trovato variazioni individuali nella suscettibilità fra le uova di una stessa deposizione, i cui limiti stanno fra di loro come 1:3.

Nei casi di intervento sperimentale di natura fisica o chimica, sono concepibili variazioni nell'intensità dell'azione su ogni individuo, e queste, anche se minime, possono ritenersi, almeno in parte, responsabili della variabilità dei risultati. Nel caso dell'ibridazione le variazioni delle condizioni ambientali non possono certo essere di tale entità da alterare sensibilmente lo sviluppo, e conviene quindi attribuire i risultati a condizioni insite nella natura stessa dei gameti.

Nel nostro caso due interpretazioni sono possibili. Si può ammettere che i gameti presentino una variabilità individuale, di carattere continuo, di qualche proprietà fisiologica, la quale, adottando l'interpretazione proposta, potrebbe manifestarsi con lievi differenze nel ritmo cinetico. Oppure si può ricorrere alla spiegazione offerta dal Newman (1923) che così si esprime "hybrid vigor is believed to be a result of a summation in the hybrid zygote of the favorable characters of the two species; hybrid weakness in the same strain of larvae is attributed to a summation in the zygote of the lethal, semilethal or unfavorable genes of both species." Su basi genetiche, aggiunge l'autore, potrebbe anche essere spiegata la formazione dei mostri, benchè questa sia il risultato di un'inibizione differenziale.

Gli elementi raccolti nel corso di questi esperimenti non sono sufficienti per decidere fra le due interpretazioni. L'essere le variazioni di carattere continuo, indurrebbe a far ritenere più verisimile una variabilità di qualche caratteristica fisiologica dei diversi individui.

Non è escluso, del resto, che questa possa essere l'espressione di condizioni genetiche. Soltanto l'esame statistico dei risultati può forse darci qualche indicazione in proposito.

Ho desiderato accennare a questi fatti, perchè la loro costanza e la loro entità sono indici di una diversità nelle condizioni dei gameti, la cui analisi non mi pare da trascurare. Sono fenomeni evidentemente collegati al problema del vigore degli ibridi, che ha attratto l'attenzione dei genetisti, soprattutto dei botanici.

VII. SUMMARY

The results of hybridization between the two species of toads *Bufo vulgaris* and *B. viridis* are very different in the reciprocal crosses.

1. The embryos of the cross *Bufo viridis* ♀ × *B. vulgaris* ♂ show a considerable retardation of the developmental rate, which is followed by extensive modifications of the ontogenesis; the rate of development appears to be correlated with the degree of abnormality. The following alterations of the normal ontogenesis were observed:

a) A small number of abnormal cleavages, which are followed by the death of the embryo in early stages. Some normal cleavages lead to the formation of "stereoblastulae" unable to gastrulate.

b) Abnormalities in the gastrulation process. They give rise to: exogastrulae, ring-embryos, "spina bifida" of various degrees, "asyntaxia blastoporalis" without spina bifida. A more or less pronounced asymmetry may arise in these forms.

c) Failure of the closure of the medullary folds, total or limited to the cephalic region. In the first case, anidian embryos arise; in the second case, embryos with complete acephalogenesis, or hemicrania.

d) Embryos which show a marked differential inhibition, especially evident in the nervous system. The whole brain may be inhibited (solid brain) or its prechordal part only, while the chordal part may be less, or not at all, altered. The body shows a characteristic dorsal bending, more or less pronounced; the rostral region (mouth, nasal pits) is strongly inhibited, as well as the heart (string heart). All such embryos die very early.

e) Embryos with less inhibition of morphogenesis, localized in the forebrain or, in the best cases, in its olfactory region. The rostral re-

gion is more or less altered, according to the degree of abnormality of the forebrain. The inhibition produces essentially a shortening of the prenasal region, instead of a tendency to the approximation and fusion of the lateral organs, as frequently occurs in other experimental conditions. The heart is always considerably deformed; hematopoiesis takes place only in the best-developed embryos, but it is always far from normal. The body form is less altered and the development proceeds farther, though considerably retarded, reaching more advanced stages than it does in the embryos considered under (*d*). These tadpoles never form the posterior limbs; all of them die with symptoms of hydropsy.

The inhibitions described above show a great similarity to those obtained by means of X-rays on the amphibian gametes or embryos. Also the cellular alterations are similar in both cases.

2. The embryos of the reciprocal cross, *Bufo vulgaris* ♀ × *B. viridis* ♂, develop much better. The following cases were recorded:

a) A very small number of early alterations, arising during the gastrulation or shortly after it, and followed by the early death of the embryos.

b) A few cases of considerably retarded development. They generally show an inhibition similar to the cases of best development recorded for the reciprocal cross. All these individuals die long before metamorphosis.

c) The great majority of embryos develop like the controls. At first a certain delay can be noted in the process of development; it is never very marked, and later on it disappears. The tadpoles have about the same size and aspect as the controls; they eat and live well until the time of metamorphosis, when a considerable mortality decimates the cultures.

d) Some of the tadpoles, a few weeks after fertilization, acquire larger size than their brothers and the controls. They are considered as cases of acceleration of development; they reach metamorphosis earlier than the others and the controls, and show a low mortality.

In the metamorphosed little toads some paternal characters are evident.

3. The causes of the varying success of these and other reciprocal crosses are discussed, and the interpretations proposed by various

authors reviewed. Attention is called particularly to the interpretation which holds the asynchrony of the developmental rhythms (and especially of the kinetic rhythms) of the two parent species as the factor chiefly responsible for the observed inhibitions. The introduction of a slower sperm seems to be more harmful than the introduction of a sperm with a faster rhythm than the egg. This interpretation does not require the assumption of any specific action of unknown substances (though it does not exclude it) and seems to agree with other cases of heterospecific hybridization among amphibians, teleosts, and echinoderms. To make a correct comparison of the two rhythms, however, it is necessary to know the initial N/P ratio of the normal zygotes.

4. The inhibition can be explained, on the basis of this interpretation, as due to an alteration of the nucleoplasmatic relationships, namely, to lack of equilibrium between the physico-chemical relations in the plasmatic colloids during the kinetic processes. The retardation of development, i.e., of the kinetic processes, correlated with the appearance of inhibitions, seems favorable to this hypothesis. Recent workers on differential inhibition produced by means of physical and chemical agents are inclined to adopt a similar interpretation.

5. The differential susceptibility is considered as due to differences in metabolic level of different parts and tissues, according to the facts demonstrated by Child's researches. Such a theory gives a satisfactory explanation of the alterations in morphogenesis, and it is the only one which is able to explain the differences in susceptibility shown by the various tissues.

6. The mechanism of formation of the observed monstrosities is briefly discussed, on the basis of the modern knowledge of amphibian development. The distinction is made between primary and secondary inhibitions, the latter being due to correlations of different nature existing between developing organs.

7. Attention is called to the fact that in each of the reciprocal crosses, a wide range of variability in the results of ontogenesis is constantly obtained. This condition is analogous to what has been described in some teleost and echinoderm hybridizations. Two interpretations of this phenomenon are possible; it may be due (1) to a

continuous individual variability of certain physiological characteristics of the gametes (which could be expressed by slight differences in the kinetic rhythms); or (2) according to Newman's hypothesis, it may be an expression of different combinations of various genetic sets of the gametes. Statistical analysis, still lacking, would probably help in deciding the alternative.

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SPIEGAZIONE DELLE TAVOLE

Sezioni trasversali, colorate con ematossilina-eosina, fotografate con apparecchio Spencer. Ingrandimento di 70 diam., tranne che per figg. 1 e 7, ingrandite di 100 diam.

Indicazioni comuni a tutte le figure:

a = vescicola, o calice ottico.	*n* = organi olfattori
c = corda	*o* = otocisti
d = diencefalo	*p* = pronefro
e = cellule epidermiche	*t* = telencefalo
h = cuore	*v* = organi adesivi

THE RESPONSE OF GUINEA PIG MAMMARY GLANDS TO INJECTED SEX HORMONES AND OVARIAN GRAFTS AND ITS BEARING ON THE PROBLEM OF SEX HORMONE ANTAGONISM[1]

(Twelve figures)

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INTRODUCTION

THE biological potentialities of the mammary glands present many interesting problems, such as their origin, their differentiation, their growth, and regulation of their normal function of lactation by hormones. The present investigation involving primarily a study of their response in growth, differentiation, and function to various hormonal conditions was undertaken with the hope of obtaining additional information relative to their response to various hormone influences.

Since Steinach (1912) introduced his concepts of the influence of sex hormones in the organism, several points of special interest have arisen. A brief review will serve to illustrate the lack of agreement among investigators as to the extent and type of sex hormone influences.

Steinach (1912) first demonstrated, chiefly by gonad grafts, that the majority of the secondary sex characters were controlled by secretions from the homologous sex organs. Thus male structures, such as the penis, seminal vesicles, prostate glands, etc., were dependent upon the testis for their state of growth and function, while female structures, including mammary glands, were largely controlled by the ovary. These conclusions have received almost universal substantiation in work since that time.

A second contention of Steinach, however, has been severely questioned. He maintained that sex hormones, in addition to stimulating homologous sex characters, inhibited the sex characters

[1] This investigation has been aided by a grant from the Committee on Research in Problems of Sex of the National Research Council; grant administered by Professor Frank R. Lillie.

of the opposite sex. This hypothesis was based in part upon the fact that gonads transplanted into normal animals of the opposite sex did not persist, nor did they show any hormal effect. On this basis he postulated a "sex antagonism" that extended to the gonad tissue itself. When an animal was previously "desexed" by removal of its gonads, subsequent or simultaneous transplantation of gonads of both sexes led to persistence and some endocrine function of each.

Sand (1918) was the first to point out the necessity of modifying Steinach's concept of antagonism. He was able to obtain persistent and functioning ovarian grafts when the ovary was transplanted into the testicle of the male guinea pig. He postulated the presence of some food substance in the blood stream necessary for normal gonad function which was removed from the blood stream and stored in the gonad. Gonads grafted intramuscularly or to the peritoneum of the opposite sex were unable to obtain this substance in sufficient amounts to become active, whereas ovaries grafted into the testis could obtain the necessary substance. Such grafts were successful in both rats and guinea pigs. In the latter the ovarian tissue was quite normal and its endocrine influence proved by growth and function of the mammary glands. In rats this ovarian endocrine activity was not demonstrated, for a rudimentary nipple is lacking in the male. These experiments are the basis for Sand's hypothesis. It should be emphasized that, in cases where endocrine activity of the ovary was revealed, one or both testes showed degenerative changes in the tubules. This was occasioned by adhesions between the testes and the abdominal or inguinal peritoneum following the operation. Sand apparently does not attach great import to this atrophy. These experiments and also those of Moore (1921*a*) and Lipschütz (1925*a*) clearly demonstrate that successful heterosexual gonad grafts can be made without previous or simultaneous castration of the host.

Lipschütz, from 1918 to 1929, has worked extensively with phenomena that have added further complications to the hormone antagonism question. He was able to transplant ovaries successfully into the kidneys of normal male guinea pigs, but such ovaries usually failed to exhibit evidence of hormone secretion or did so only after a very long time. Thus ovaries placed in the kidney of a cas-

trated male or female animal gave evidence of endocrine activity within a period of approximately two weeks, for at this time the mammary glands of the host began a rapid growth that continued perhaps for some months. In the kidney of the normal male, however, ovarian grafts though well incorporated did not stimulate growth of the mammary glands until a long period of latency had intervened. Experiments conducted by Lipschütz in Esthonia placed this period of latency of mammary hypertrophy in normal males bearing ovarian grafts at from five to eight months (1925*a*), but later ones done after his arrival in Chile reduced this period of latency to approximately four to seven weeks (1927*b*). Such delayed endocrine function on the part of ovarian transplants was considered to be due to an antagonistic influence from the testes.

Further evidence for hormone antagonism was found by Lipschütz (1925*b*) in the sudden appearance of mammary gland growth following castration. Thus a normal male might be host to an intra-renal ovarian graft for a period of five months without external evidence of its presence, but, if the testes were removed, mammary growth would become evident within two weeks and growth would continue for several months. Lipschütz termed such a response the "unbolting reaction," visualizing that removal of the testis suddenly released the ovarian graft from its suppression and permitted its function. However, removal of the testis was not essential as either reduction in mass or elevation of testes into the abdomen (experimental cryptorchidism) served equally well to release the ovarian graft from the antagonistic influence of the normal testicle.

It should be pointed out that the differences of opinion just reviewed came from observations derived from studies in which the surgical method of treatment was almost exclusively used. The utilization of purified hormone preparations, it would seem, should lead to more nearly similar ideas, but it has not proved so. Thus Steinach has recently renewed his earlier contentions of sex hormone antagonism to interpret the results of injecting ovarian extracts into male animals (Steinach and Kun, 1926 and 1931). Normal male rats injected with the female sex hormone (oestrin, menformon, etc.) were found to exhibit not only damage to the testes but a degenerate condition in all the reproductive accessory organs.

Moore (1919, 1920, 1921*a*, *b*, 1930*a*) has denied repeatedly that conclusive evidence has been presented for antagonism between the two sex glands. In a long series of experiments he has been able to show for both sexes of rats and guinea pigs that heterosexual gonad transplants were easily obtained when the host's own gonads were present. Testis grafts were recovered months after transplantation into normal females and were carrying on spermatogenetic activity despite the fact that such females had mated, delivered, and suckled young. Similarly, ovarian grafts showing normal follicular development were recovered from males that had inseminated females during the period of graft persistence.

Moore and Price (1932), while in entire agreement with others that the female hormone (oestrin) causes damage to the male reproductive tract when administered through subcutaneous injection, pointed out that administration of testis hormone to normal young males induced testicular damage of a similar grade and character to that which followed oestrin treatment; they hold that the condition developed after each treatment must have a common basis of explanation and such a basis cannot rest upon an antagonism between the two sex hormones. It was shown by them that a given dosage of testis hormone just sufficient to maintain the male accessory reproductive organs in a normal condition would have entirely similar effects if injected alone, or accompanied by a dose of oestrin previously found highly injurious to the testes of males. Rather than an antagonism between the two hormones Moore and Price explain their results on the basis of a reciprocal gonad-hypophysial interaction. Oestrin is injurious to the male reproductive tract not because it is antagonistic to the male system or secretions from it but rather because oestrin suppresses the activity of the hypophysis in such a manner that the organism is no longer provided with the necessary hypophysial secretions for gonad maintenance and function, hence damage to the male system by oestrin is similar to that which follows hypophysectomy. Their contentions are supported by numerous lines of evidence.

In view of the radically different existing opinions, further investigations in the field of hormonal influences within the organism are to be desired. In the following pages will be given the results of an

investigation carried on for the past three years in which the reactions of the mammary glands of the guinea pig to various hormonal states within the organism have been followed. The ordinary surgical treatments and the injection of hormone preparations have been combined in an attempt to define the limits of response of these structures to the different conditions imposed upon them.[2]

MAMMARY GLAND RESPONSES IN NORMAL AND GONADECTOMIZED GUINEA PIGS

The mammary glands though most often considered as secondary sexual characters of the female are differentiated equally in both sexes of the guinea pig. At birth in either the male or female the nipples are well defined and approximately 1 millimeter in height. The gland tissue proper consists of branched tubules located in the dermis of the skin and has a circular spread of approximately 1–2 centimeters in diameter. Histologically the tissue of these structures is as abundant and as well developed in the male as in the female.

In normal females repeated measurements of the nipples show clearly that a slow growth occurs from birth to puberty. At the time of the first oestrum, or perhaps a few days earlier, the nipples become quite turgid and from this time a more rapid growth ensues. At six months of age their height is of the order of 4 or 5 millimeters and little further increase is evident excepting during pregnancy and

[2] This problem was suggested to me by Dr. Carl R. Moore, and it is with great pleasure I acknowledge my indebtedness to him not only for a most interesting problem but also for his constant guidance and encouragement.

The material requirements for an investigation of this nature are numerous, and the investigation itself could not have been conducted without the hearty co-operation of others. To Professor R. G. Gustavson, of the Department of Physiological Chemistry of the University of Chicago and the University of Denver, I am indebted for preparations of the female hormone, oestrin, prepared from human placentae and human pregnancy urine. The preparations had been carefully assayed by the method outlined by Coward and Burn (1927). To Professor F. C. Koch and Dr. T. F. Gallagher, Department of Physiological Chemistry of this University, I am indebted for preparations of testis hormone prepared from extracts of bull testes and carefully assayed by Dr. Gallagher by the capon comb method (see Gallagher and Koch, 1930). I also wish to thank Dr. O. Kamm of Parke Davis Company for preparations of anterior pituitary extracts. A great number of the 500 guinea pigs employed in this study were kindly placed at my disposal by Dr. Sewall Wright from his large colony maintained in connection with experiments in genetics. It is indeed a pleasure to express my gratitude for these invaluable aids.

lactation (see Fig. 1). The hormones of pregnancy cause marked changes both in the nipple height and in glandular differentiation. Though a great deal of variation is found in nipple height of pregnant females the usual state is one of approximately 8–10 millimeters. During the active nursing period this height is increased, probably due to the mechanical factors in suckling rather than to hormonal ones. After weaning a decided regression occurs, but usually the nipples of females that have given birth to young remain at a level above those of virgin animals.

The mammary glands of young males experience a similar slow growth to those of females up to the stage of puberty, but there is no

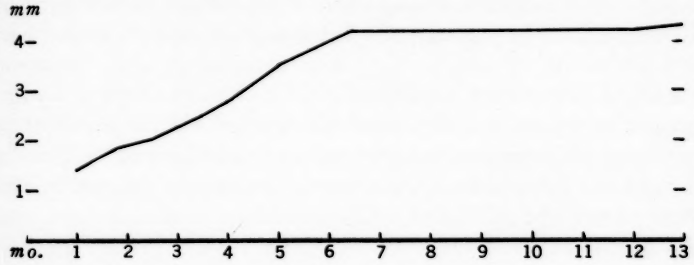

FIG. 1.—Growth of nipples of normal females. Each point is an average of 35 individuals.

marked increase either in nipple height or growth of the secreting portion beginning at that time. Neither is there any evidence of regression as the testes become functional.

Removal of the ovaries introduces marked differences in the condition of the mammary glands of the females. When ovariotomy is performed before the stage of puberty is reached there is no evidence of a growth acceleration either in nipples or in the tubules or acini of the glandular portion. In fact, the conditions are entirely comparable to those found in the normal male. When the ovaries are removed at the age of six months, when sexual maturity has been reached, the regression occasioned in the glandular tissue is much more rapid than that of the nipples; the latter may fail to exhibit regression for a period of four to five months.

Removal of the testes occasions no changes in the mammary ap-

paratus of males. The glands or nipples of prepubertally castrated males differ in no appreciable manner from those of normal males. Castration in adults likewise fails to introduce any modification in these structures.

It therefore becomes apparent that the mammary gland of the guinea pig is a somatic structure, present at birth in both sexes, that fails to exhibit any evidence of sexual dimorphism. Its growth and further development is stimulated by hormones derived from the ovary, but it appears to be insensible to hormones from the testis. Since prepubertal castration fails to introduce changes in its structure, it is evident that the rudimentary condition found in the males is due only to lack of substances that will stimulate its development, rather than to the presence of substances that arise from the testis and inhibit its development. Early ovariotomy and castration renders the mammary apparatus of both sexes identical so far as I am able to determine. Structurally it appears that the glands of the two sexes are equipotential, but recourse must be had to injection of hormone preparations before one is justified in concluding that they possess a physiological equipotentiality.

MAMMARY GLAND RESPONSE TO HORMONE INJECTIONS

EQUIPOTENTIALITIES

My chief interest in a study of the control of the mammary glands concerns not only the question of their equipotentiality in the two sexes but also whether their potentialities are in any manner affected by hormones from the testis. It was early appreciated that information regarding either problem would require some approach to quantitative methods of study.

Three methods of study of the mammary glands have been investigated and all applied to some extent; in some cases all three have been utilized for the same material. A histological study of the glandular tissue has some advantages and many disadvantages. Whereas the study of sectioned material shows more clearly the actual state of the secreting tissue, and one can readily distinguish the normal female gland from that of the normal male, still it is with great difficulty that one can apply quantitative methods to dis-

tinguish different grades of activity. The study of the dissected gland as a whole mount preparation has certain advantages over that of sectioned material. Thus when the gland is fixed in 10 per cent formalin and stained *in toto* with dilute haematoxylin the glandular tree is very apparent among the fat and connective tissue. However, despite the fact that the spread of the glandular portion gives a more adequate comparative picture, it is difficult to obtain any satisfactory measure of the response because of the lack of compactness of the tissue. The most satisfactory index of activity proved to be the mere measurement of the nipple height. It was found that nipple height paralleled to a large degree the glandular differentiation, and since it could be repeatedly taken without sacrifice of the animal, or a single gland, it has served as the chief index of response.

In order to compare the reaction of the mammary glands of males and females, it required first to ascertain the threshold of response to oestrin. In preliminary experiments, therefore, different dosages of oestrin were injected subcutaneously each day for twenty days into adult castrated male animals. Since the glands had remained entirely quiescent from birth, and since the testis hormones were eliminated through castration, the response of the glands would start from a base level. Measurements of nipple height were taken throughout the experiment and the gland tissue was preserved and studied in histological preparations. The results obtained from this preliminary investigation revealed the fact that a definite response in growth of the mammary apparatus occurred with a daily injection of much less than one rat unit of oestrin, the rat unit corresponding to the Coward and Burn designation (1927).

Fifteen adult male guinea pigs, castrated from four to six weeks preceding a more detailed experiment, were divided into lots of 3 each. These lots were given daily injections of (*a*) olive oil, (*b*) 0.05 rat unit of oestrin, (*c*) 0.125 rat unit of oestrin, (*d*) 0.25 rat unit of oestrin, and (*e*) 0.375 rat unit of oestrin. The samples were so arranged that each animal received the same quantity of olive oil as the control group. Injections were continued for twenty days during which time nipple height was repeatedly determined and the mammary gland tissue was preserved and sectioned at the termination

of the experiment. The results obtained from nipple height measurement are shown in Figure 2.

The sectioned glands were of little value in determining relative responses to these small dosages of oestrin but were of general confirmative value for the nipple response. Examination of the figure, however, shows the definite responses given by nipple growth. Daily injection of 0.05 rat unit of oestrin is the minimal amount to which a response is obtained, whereas dosages of 0.125 and 0.25 rat unit produce perfectly definite growth responses. With a knowl-

FIG. 2.—Growth response of nipples of adult castrate males to 20 daily injections of oestrin. Each point represents the average percentage of increase in nipple height of three animals to a given concentration of oestrin injected. The dosages are .05 .125, .25, and .375 rat unit.

edge of such responses on the part of the glands from castrated males, a comparative study was made of the relative sensitivity of these structures in the two sexes.

A group of 18 males and 18 females were subjected to gonadectomy before thirty days of age and permitted to grow to adults. They were then divided into three groups consisting each of 6 males and 6 females. Group (*a*) was given daily injections of 0.125 rat unit of oestrin, group (*b*) 0.25 rat unit, and group (*c*) 0.5 rat unit of oestrin. The experiment was carried through a period of thirty-five days, and nipple measurements were taken regularly (see Fig. 3).

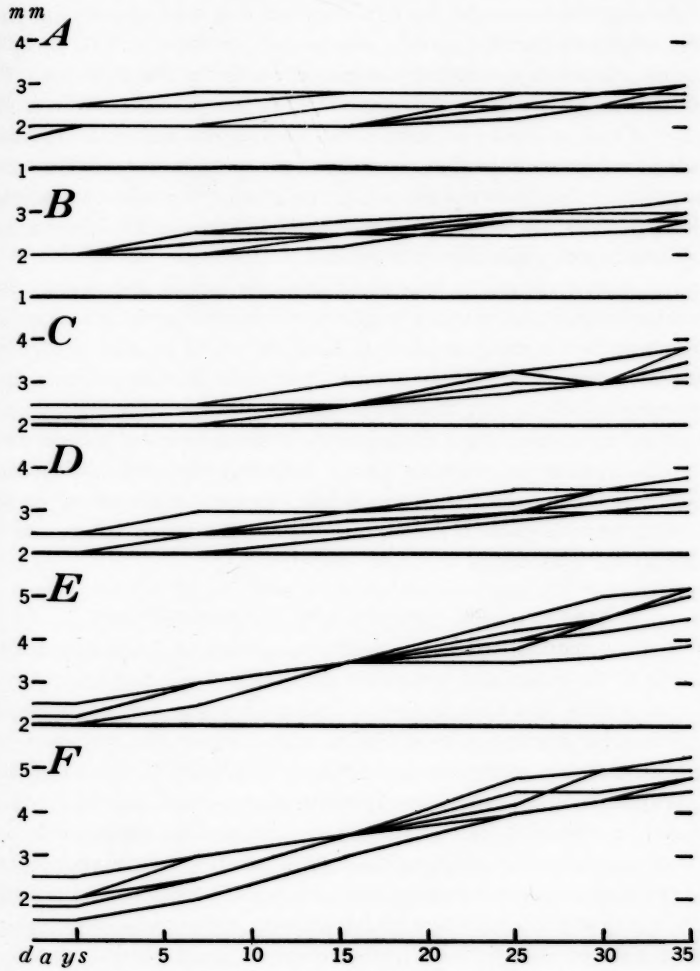

FIG. 3.—Growth response of nipples of adult castrated males and spayed females (prepubertal gonadectomies) to various concentrations of oestrin injected for 35 days. *A*, six spayed females receiving .125 rat unit daily; *B*, six castrated males receiving .125 rat unit daily; *C*, six spayed females receiving .25 rat unit daily; *D*, six castrated males receiving .25 rat unit daily; *E*, six spayed females receiving .5 rat unit daily; *F*, six castrated males receiving .5 rat unit daily.

An examination of the figure reveals the fact that nipple height as recorded runs parallel to the amount of oestrin injected. It is a simple matter to determine the groups receiving the different dosages; although some variability among the groups was noted it is always of such an order as to be of no other significance than minute individual variability. Of particular importance is the fact that the response of the male nipples was in no manner different from that of the females; the nipple response of males is just as sensitive to the injected oestrin as is that of females.

The data presented in this and the preceding section permit the conclusion that the mammary gland of the guinea pig is a somatic structure that is equipotential, anatomically and physiologically in the two sexes. It is affected by, and responds to, oestrin but is apparently unaffected by hormones from the testis. The young animals do not exhibit a sex dimorphism in the mammary glands, and the dimorphism expressed in adults is merely the result of the response of the female glands to oestrin; the lack of response on the part of the male gland is due to the lack of ovarian secretions rather than to the presence of testicular secretions.

MAMMARY GLAND RESPONSE TO SEX HORMONE MIXTURES

It was demonstrated in the preceding section that the mammary glands of the males and females were equally sensitive to the injection of oestrin. For the purpose of determining whether the presence of testicular hormones modified in any manner the response to oestrin, the mammary changes were studied in both males and females that received injections of oestrin alone and of oestrin accompanied by effective doses of testis hormone. The experiment involved the utilization of 32 animals all of which were twelve months old; 11 males and 12 females of this group had been gonadectomized before puberty. The groups and treatment were as follows: (1) 3 normal males—oestrin and testis hormone, (2) 3 normal males—oestrin alone, (3) 4 castrated males—oestrin and testis hormone, (4) 4 castrated males—oestrin alone, (5) 4 spayed females—oestrin and testis hormone, (6) 4 spayed females—oestrin alone, (7) 3 castrated males—olive oil, (8) 3 normal males—olive oil, and (9) 4 spayed females—olive oil. Each animal was given daily injections for a

period of twenty days and the injected material was so compounded that each animal received the same quantity of olive oil as the control group injected with olive oil alone. The quantity of oestrin was in every case 0.2 rat unit daily but those animals receiving the mixed hormone injections had in addition seven bird units of testis hormone. This amount of testis hormone had been shown previously to be a dose effectively substituting for the testis (Moore and Gallagher, 1930; Moore and McGee, 1928). Moreover, its potency was proved in these experiments by the fact that the prepubertally castrated males, when a year old, responded on the tenth day of the mixed hormone injections by giving a coagulable ejaculate after electrical stimulation on the head (for the method see Moore and Gallagher, 1930). Nipple measurements were made before injections were begun and on each five succeeding days. At the end of the twenty days of injection the mammary glands removed for sectioning afforded material that confirmed the response indicated externally in nipple growth. The observations on nipple measurements are expressed in Figure 4 as averages for the different groups involved.

A study of the figure reveals several points of prime importance which were confirmed by a study of the sections of these glands.

In every case, considering the groups as a whole, the mammary glands of animals (male or female) receiving effective doses of testis hormone along with the oestrin showed as great nipple growth as did those receiving the same dosage of oestrin alone. In fact, two of the groups showed slightly more growth after the administration of both hormones. Since the mixture of the two hormones was made at the beginning of the experiment, sufficient time was given for the occurrence of any chemical interaction that might be conceived possible; hence the absence of any modification of the potencies of oestrin by the testis hormone indicates clearly the absence of chemical antagonism between these two substances. Furthermore, the fact that responses of the mammary apparatus were as great in the presence of effective doses of testis hormone as when oestrin alone was administered proves beyond doubt that the testis hormone is in no manner a deterrent to the responses of the glands to the homologous hormone stimulation.

The conclusion is justified, therefore, that sex hormone antagonism does not exist if one may understand antagonism to mean either a chemical interference or neutralization between the two substances or the interference of one upon the normal reaction of the end organ to its homologous hormone. Rather does the end organ (in this case the mammary glands) respond to its normal stimulating substance, or homologous secretion, and exhibit no response to the heterologous secretions either as a stimulating effect or an inhibiting effect.

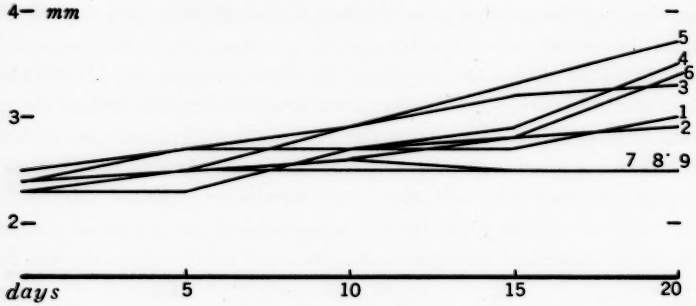

FIG. 4.—Growth response of the nipples of adult normal and castrated males and spayed females to oestrin and mixtures of oestrin and testis hormone. Animals receiving oestrin only were injected with .2 rat unit daily. Those receiving the mixture were injected with .2 rat unit of oestrin plus 7 bird units of male hormone in olive oil daily. A third group received only olive oil. 1, Three normal males receiving oestrin and testis hormone; 2, Three normal males receiving oestrin; 3, Four castrated males receiving oestrin and testis hormone; 4, Four castrated males receiving oestrin; 5, Four spayed females receiving oestrin plus testis hormone; 6, Four spayed females receiving oestrin; 7, Four castrated males receiving olive oil; 8, Three normal males receiving olive oil; 9, Four spayed females receiving olive oil.

MAMMARY GLAND RESPONSES TO OVARIAN GRAFTS

The preceding sections have presented observations that are fundamental in an interpretation of the responses of the mammary glands in both sexes to the stimulating action of oestrin. The chief difficulty in understanding completely the contentions of those who maintain the view of an antagonistic action between the hormones involves the interpretations of observations made as a result of ovarian transplantation. It appeared desirable, therefore, to reinvestigate the problem of the effects of ovarian grafts in different types of animals.

The method employed in making ovarian grafts for this investigation is a modification of the one first described by Marshall and Jolly (1907) and later employed by Lipschütz (1925*a*); this involves for the most part the transplantation of an ovary into the kidney where a particularly good vascular bed materially aids the establishment of vascularity. After clipping, shaving, and sterilization, an incision is made through the skin lateral to the mid-dorsal line. Epaxial and hypaxial muscles are separated and the dorsal portion of the kidney exposed. The kidney is slightly elevated by inserting a needle into its substance; and the ovarian tissue, threaded bead-like on short lengths of No. 00 catgut, is drawn against the kidney tissue, the needle passing tangentially through a small portion of the organ. An incision is made with a narrow scalpel blade quite deeply into the kidney alongside the needle, and the ovary, previously scarified with a needle point to aid blood-vessel ingrowth, is pulled into the substance of the kidney where it is held firmly by traction of the catgut. Removal of the needles, return of the kidney to its normal position, and closure of the sutures completes the operation which with practice can be performed in approximately ten minutes. The donor ovaries were obtained from sacrifice of normal healthy females, or at simultaneous operation, and were never separated from the donor for more than a few minutes previous to incorporation in the host. The amount of ovarian tissue transplanted has varied from one-half to two entire ovaries, but no significant difference has followed the variable amount implanted; hence they will be treated as if a single whole ovary had been utilized.

Ovaries have been transplanted into more than 270 animals, and these animals have been carefully examined for periods usually of from five to six months; some, however, were under observation for more than a year. The reactions of the mammary glands have been followed closely and the nipple height recorded frequently throughout the course of the experiment.

OVARIAN GRAFTS IN ADULT NORMAL MALES

Intrarenal ovarian grafts.—Ovaries have been transplanted into 150 adult normal males. Ninety-five per cent of these host males failed to show any mammary gland hypertrophy over a period of four to six months. Subsequent to this period 100 of the males were

subjected to some testicular manipulation. Among these, 75 involved the transfer of the testes from the scrotum into the abdomen (experimental cryptorchidism) and 25 involved castration. Twenty of the 100 responded immediately by increased growth of the nipples. A few examples of these responses to illustrate the various reactions observed appear advisable.

Table I will serve to illustrate the type of response occurring after delayed castration.

TABLE I

PROTOCOL OF A NORMAL MALE (NO. 475) HOST TO AN OVARIAN GRAFT

Nipple Height in Millimeters		Date	Remarks
Left	Right		
2.5	2+	3–19–31	Received intrarenal ovarian graft.
2.5	2+	4–10–31	Donor 7 months old. Host 8 months old
2.5	2+	5–30–31	
2.5	2+	7–17–31	
2.5	2+	8–13–31	Castrated. Testes normal, motile sperm in epididymis
2.5	2+	8–20–31	
8.0	8.0	8–31–31	
13.0	12.0	10–31–31	
13.0	12.0	2– 4–32	Died. Ovarian graft preserved.

Variations in nipple response are to be expected, and were noted; the observations in Table I are characteristic for this general type of response. A slow growth of the nipples in normal males occurs from birth to maturity but this growth is very slight in comparison with that occurring in the normal female. Since a similar growth occurs in castrated males or females, it is to be attributed to general body growth rather than to a response to hormonal influences. In the above-cited case the normal male eight months old showed a nipple height of approximately 2 millimeters. For a period of five months after ovarian transplantation the nipples failed to give evidence of the presence of the graft. Within two weeks after removal of the testis, however, the nipples had increased from a height of 2 millimeters to 8 millimeters, and continued to grow for a period of two months, finally reaching a height of 13 millimeters.

In such cases as this, therefore, the absence of mammary reactions cannot be attributed to the failure of incorporation of the graft.

That it was present and capable of function is attested by the fact that immediately upon testis removal mammary growth began and progressed rapidly. It should be further noted that virgin females of similar age exhibit nipple heights of approximately 4–5 millimeters whereas the male cited above produced nipples 13 millimeters in height. It becomes clear, therefore, that the presence of the testis in some manner interferes with the endocrine secretory function of a well-incorporated ovarian graft. This type of reaction has been termed the "unbolting reaction" by Lipschütz (1925*a* and *b*) and was considered by him to give evidence of an antagonistic action of the testis upon the ovarian graft. But in the previous sections evidence has been presented that an antagonism between the internal secretions, as we have used these terms previously, is in no way indicated. Some other explanation must be given to account for the conditions observed.

Among the group of normal males receiving ovarian grafts, 6 males were observed to experience nipple hypertrophy with but little interference by the testes. Nipples began a slow growth in these 6 animals at varying times after ovarian transplantation, but growth was neither so rapid nor so pronounced as that occurring after castration. Evidently whatever influence the testes exert in preventing the activity of the graft was only partially operative; nipple height in such cases was never greater than 7 millimeters. At autopsy the testes of such males were entirely normal in appearance and histology, and the male accessory organs were fully developed. The testes were active both in gametogenesis and in hormone production. The protocol given in Table II illustrates one such case.

A second type of testicular manipulation was the removal of the testes from the scrotum and confinement within the abdomen. Such an operation in male guinea pigs is invariably followed by marked degeneration of the testes and the ultimate loss of all the gametogenetic portion of the testis, but no modification of the endocrine activity of the organ occurs (see Moore, 1924). Lipschütz (1925*a*) has reported such a testis manipulation to be equally as effective as castration in the removal of the inhibiting influences of the testicle on an ovarian graft.

Among the group of normal males bearing intrarenal grafts, 36

animals, which for months had failed to exhibit mammary responses to the ovarian grafts, were subjected to the above type of operation. Of this number 12 showed an immediate response of nipple growth and those which failed to respond were later castrated, also without a response; search for the grafts at a slightly later period showed them to be degenerate and represented only by connective tissue.

These cases confirm the contentions of Lipschütz and others that loss of the germinal portion of the testis through abdominal elevation removed the influence from the testis that interfered with

TABLE II

PROTOCOL OF A NORMAL MALE (NO. 357) HOST TO AN OVARIAN GRAFT

Nipple Height in Millimeters		Date	Remarks
Left	Right		
1.5	1.5	10– 6–30	Received intrarenal ovarian graft. Host 6 months old. Donor 50–60 days old
4.0	4.5	11–18–30	
6.5	6.5	12–10–30	
7.0	7.0	12–23–30	
6.5	6.5	1– 7–31	Sired normal litter
6.0	6.0	1–23–31	

ovarian endocrine function. However, the loss of the germinal epithelium in no manner interferes with the hormone secretion of the testis, at least with that portion of the hormone that controls the male accessory reproductive organs; it is obvious, therefore, that further interpretations are necessary (see discussion).

Intra-testicular ovarian grafts.—Sand (1918) transplanted ovaries into the testes of guinea pigs and noted their endocrine influence upon the mammary glands, but a careful survey of these protocols indicates that, in every case in which the ovarian graft was functional, testicular degeneration was also involved.

During the course of my study, operations were made in which ovaries were placed within the testicular substance in 23 normal adult males. The operation must be carefully done if one wishes to avoid the complications of testis degeneration due to adhesions and approximation of the testis to the higher temperatures of the abdomen, which are injurious to the germinal epithelium. In all cases

the testes were drawn from the scrotum through a low median ventral incision in the body wall. A small incision through the *tunica albuginea* permitted insertion of an ovary into each testis. The subsequent careful repair and the return of the testis to the uninjured scrotum resulted in a few cases in which no adhesions or scrotal disturbances were involved.

In all cases where the testes remained within the scrotum without adhesions and without injury to the germinal epithelium, the mammary gland response was negative. Subsequent elevation of the testes containing the ovary grafts from the scrotum into the abdomen, however, brought forth the mammary response in every case in which a graft could be recovered at autopsy. Furthermore, all graft-bearing animals in which the operation led to the formation of adhesions and moderate injury of the gametogenetic tissues of the testis showed pronounced mammary growth.

From these data it becomes clear that ovarian grafts in the kidney, or testis, of normal adult males become successfully incorporated and persist for months without being able to secrete hormone. That this hormone is not being produced or released, rather than that it is destroyed or rendered ineffective, is shown by the fact that injections of minute quantities of the hormone subcutaneously into normal males bring on immediate responses of the mammary apparatus. Conditions that lead to injury or loss of the germinal epithelium of the testis such as castration, or experimental cryptorchidism, make it possible for a graft, which has long remained inactive, to become functional in an endocrine sense almost at once.

Despite the enormous increase in the mammary gland tissue and nipples, there were no cases among the adult normal males in which lactation occurred.

OVARIAN GRAFTS IN CASTRATED MALES

It was shown in the preceding section that ovarian grafts in normal male animals usually failed to exhibit any endocrine function despite the successful incorporation of the graft. In a few cases, slight mammary hypertrophy occurred after some delay. Invariably, when graft incorporation was successful, testis removal or the induction of some injury permitted a response in the mammary

tissue. In comparison with this result, that obtained after transplantation of ovaries into castrated males is markedly different.

Forty-five young males were castrated at thirty days of age and when they had attained an age of five to six months one ovary from an adult female was grafted intrarenally into each. In no cases during the two weeks immediately succeeding transplantation was there any appreciable growth of nipples. During the second two weeks following transplantation 50 per cent of these castrated males exhibited appreciable mammary growth. All animals were carefully followed for a period of months, with frequent nipple measurements, but there were no cases in which mammary growth occurred that was not perfectly definite within the second two weeks. Upon examination at autopsy all males that had failed to exhibit mammary responses were without ovarian grafts; the site of transplantation was usually marked by scar tissue, but the graft had undergone resorption in all cases.

The record of nipple growth in a few selected cases of this group is presented in graphic form (Fig. 5). It is to be noted that nipple height reached 15 millimeters in some animals within a period of four months after transplantation. This is to be contrasted with a nipple height in adult normal virgin females of approximately 5 millimeters. It is an interesting fact that mammary growth is so much greater in these castrated males bearing ovarian grafts than in normal females, and this point is important in attempting an analysis of the activities of an ovarian graft in different animals. But despite the excessively developed nipples and the hypertrophy of glandular tissues, lactation occurred in only one of these castrated males and secretion was scanty in this case. Lactation under such conditions has been treated as the usual occurrence by several authors—Lipschütz (1926), Sand, (1922*c*), Athias (1916), Pettinari (1925), Steinach (1913, 1916), etc.—but such contentions are not in agreement with my own observations. This discrepancy will be dealt with more fully in a following section.

In conclusion, it is definitely shown that ovarian grafts are readily incorporated in a castrated male host and that mammary response is very rapid. Larger amounts of oestrin are produced by ovarian grafts in castrated males than in normal males, and even larger

amounts than in ovaries of normal female animals, as evinced by the much greater growth of nipples.

OVARIAN GRAFTS IN EXPERIMENTAL CRYPTORCHID MALES

In a preceding section it was shown that when normal male guinea pigs carried intrarenal ovarian grafts that failed to liberate

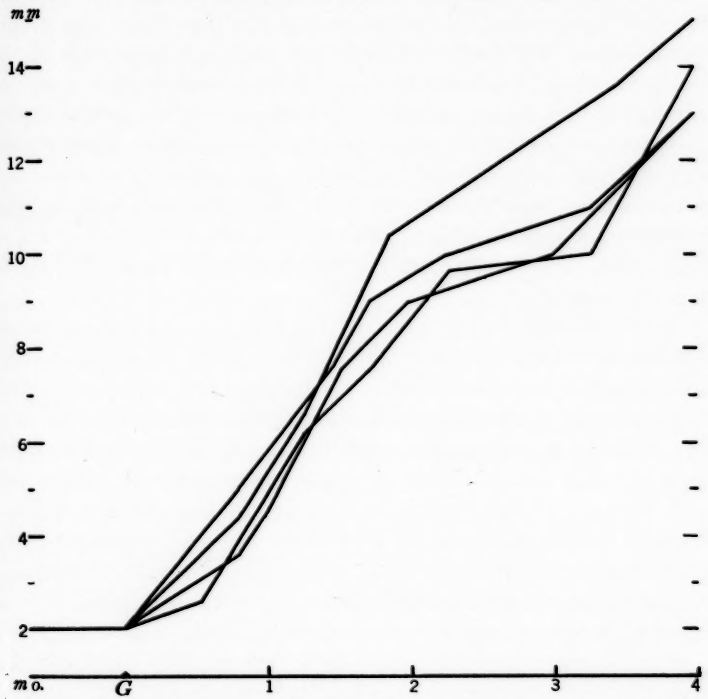

FIG. 5.—Growth response of the nipples of adult, prepubertally castrated males to ovarian grafts.

demonstrable quantities of oestrin, such a liberation occurred very shortly after elevation of the normal testes into the abdomen. In such an example of his "unbolting reaction" Lipschütz (1926) postulated the possible release of products from germinal epithelium destruction that might in some manner be responsible for removal of the inhibition exercised by the normal testicle. Since the question

deserved further consideration it appeared desirable to study the graft reactions in animals that had existed for long periods of time with testes in their final degenerate condition, rather than during the rapid and severe involution of the germinal epithelium.

Forty young males of thirty to forty days of age were subjected to experimental cryptorchidism. The testes were removed from the scrotum by severing the gubernaculum and the mesentery of the vas deferens, and were confined in the abdomen by closure of the inguinal canals. The animals received intrarenal ovarian grafts at the age of five to six months. The testes at such a period of abdominal confinement are small, usually totally devoid of germ cells, and it should be mentioned that abdominal confinement at the age given insures that the animal had never possessed a completed germinal epithelium; such animals never produce spermatozoa and the tubules of the testis contain only Sertoli cells. Interstitial tissue remains unaffected.

Approximately 60 per cent of this group of 40 males showed definite mammary hypertrophy within a period of fourteen days after ovarian transplantation. This hypertrophy was pronounced and rapid and reached its zenith within approximately two and one-half months. A few selected cases are shown in Figure 6 to illustrate the rate and intensity of nipple hypertrophy. A comparison with Figure 5 (ovarian grafts in castrated males) will demonstrate the very close similarity of the growth curves; one can quite successfully superimpose the figure of one group upon the other. And again, despite the enormous mammary hypertrophy, there were no cases in which lactation occurred.

The presence of an abdominal testis, therefore, is without effect upon the function of the ovarian grafts since essentially identical results are obtained from transplantation into castrated males and males possessing testes confined to the abdomen for a period of months. The common factor in these two groups is the absence of a germinal epithelium, however, and not the absence of testis hormone. When one examines the accessory reproductive organs of the experimental cryptorchid males bearing functional ovary grafts, it is found that they are normal in size and in storage of secretion. Moore and Gallagher (1930) demonstrated by means of the electric ejacu-

lation test that experimental cryptorchid males produced as large quantities of ejaculate as do normal males, and Jeffries (1931) showed that experimental cryptorchid rats possessed entirely normal accessory reproductive organs.

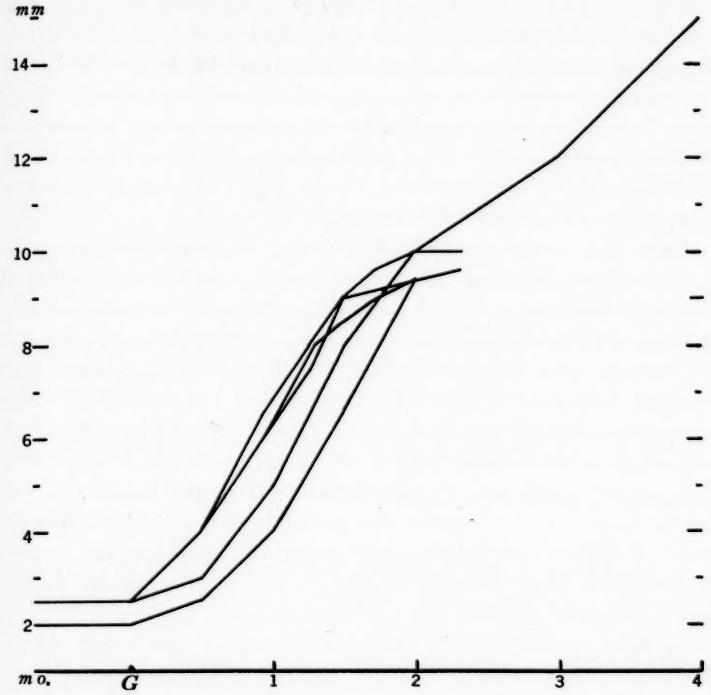

FIG. 6.—Growth response of the nipples of experimental cryptorchid males to ovarian grafts. Testis operations were made prepubertally.

It is certain, therefore, that the inhibitory influence of the testis on an ovarian graft is in no measure due to the secretions it produces that are effective in controlling the accessory reproductive organs.

OVARIAN GRAFTS IN SPAYED FEMALES

The fact that mammary glands in castrated males and experimental cryptorchid males bearing ovarian grafts were much larger

than those of normal virgin females (see previous sections) introduced the question of whether the cause lay in a different soma or in a different reactive capacity of a grafted and normal ovary. The question should be answered by the utilization of a female soma as nearly comparable to the males employed as is possible.

Thirty-five female guinea pigs were subjected to bilateral ovariotomy when thirty days of age, and when they had reached an age of five to six months ovaries were transplanted into the kidneys. They were thus as nearly comparable to the castrated males as was possible inasmuch as they were gonadectomized and received transplants at similar ages, and the nipples had remained in an undeveloped state equivalent to that of the males.

From the 35 spayed females receiving ovarian transplants, 20 showed a stimulation of the mammary apparatus within a period of twelve to fourteen days; all showing a mammary response at any time gave a definite measurable increase in nipple height within the first month after transplantation. The nipples became turgid and vascular, and grew rapidly for a short period, but soon growth was less active and they remained greatly underdeveloped in comparison with those of the treated males. The extreme nipple height noted in castrated males was 15 millimeters whereas the greatest nipple height in the spayed females was 7 millimeters. Normal virgin females of similar ages with a nipple height of approximately 5 millimeters show that, whereas nipple growth in the ovarian transplanted, spayed females was greater than normal, it was yet much below that attained by the castrated, ovarian transplanted males. The results as expressed in Figure 7 from a few selected cases should be compared with Figures 5 and 6. The causes of these peculiar differences are discussed in a later section.

Oestrous cycles.—It should be of interest to mention the oestrous behavior of these spayed females bearing functional ovarian grafts. The vaginal membrane opened for the first time just at the onset of mammary hypertrophy, twelve to fourteen days after transplantation. Vaginal smears taken twice daily according to the technique of Stockard and Papanicolaou (1917) were carefully compared with smears made from a group of normal females under observation at the same period.

The oestrous cycles of these experimental females were characterized by long periods of oestrum; this had a duration in some cases of forty-eight hours in comparison with a length of two to four hours in the normal animal. The cycles were also marked by a great deal of irregularity as can be seen from Figure 8.

The cause of such marked irregularities in the oestrous cycle may be due in part to the trauma incident to transplantation, as shown

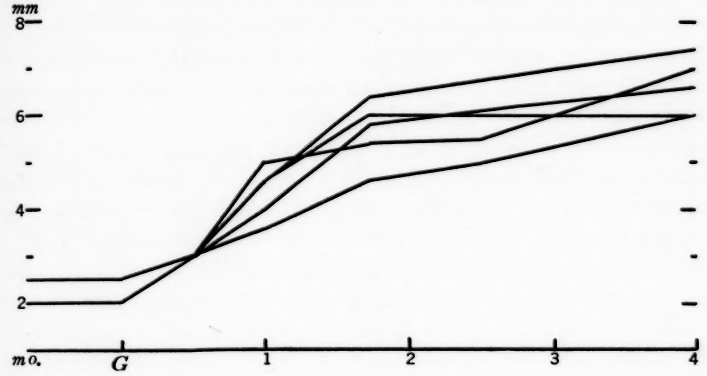

FIG. 7.—Growth response of the nipples of adult prepubertally spayed females to ovarian grafts.

by Wang and Guttmacher (1927) for the rat; they determined that injuries produced surgically on normal ovaries led to a modification of the cycles. However, it is probable that the irregularities depend for the most part upon the influences operative in causing the transplanted ovary to secrete more oestrin.

From this group of observations one may justly conclude that the functional ovarian graft in the kidney of spayed female guinea pigs secretes more oestrin and thus causes a greater mammary hypertrophy than does the normal ovary *in situ* but that it secretes less hormone and thus induces less mammary response than similar grafts in the kidney of castrated and cryptorchid males.

PERIODIC FUNCTION OF THE OVARY IN THE MALE

It is not only an interesting question but a fundamental one to inquire into the causes of greater hormone secretion on the part of an

ovarian graft in a male soma than that by a similar graft in a female soma. Lipschütz (1926, 1927*a*) suggested that ovarian grafts in the male, since they did not produce corpora lutea, were continuously active instead of periodically active as in the female. Due to this continuous activity the male experienced a condition equivalent to one of continuous oestrus and continuous hormone secretion, which was the fundamental cause underlying the greater mammary hyper-

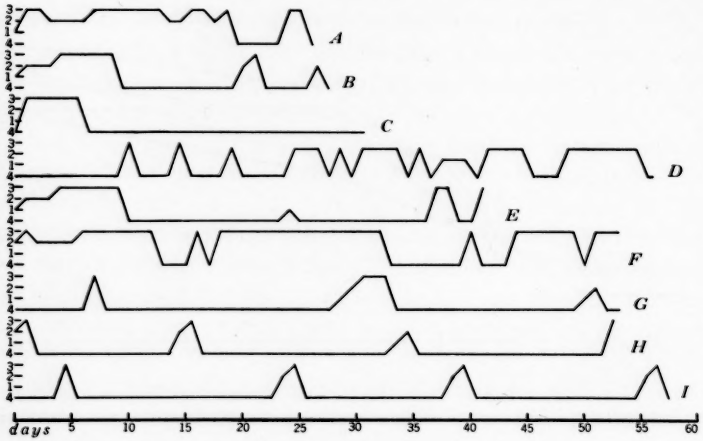

FIG. 8.—Oestrous cycles of spayed females bearing ovarian grafts. *A*, *B*, and *E* show the oestrous cycles at the beginning of activity of the graft. *C*, *D*, *F*, and *G* show the oestrous cycles from 1 to 2 months after the graft first became active. *H* and *I* show the oestrous cycle of unilaterally spayed females. These cycles are normal: (1) nucleated epithelial cells; (2) cornified epithelial cells; (3) nucleated, cornified cells, and leucocytes or cornified cells and leucocytes; (4) leucocytes only or vaginal membrane closed.

trophy. It was of interest to me to learn whether such an explanation was adequate or whether other factors would be involved in the correct explanation.

Two methods suggested themselves as offering a possible solution to the question of the periodicity of ovarian grafts in the male. The first was based upon the changes occurring in endometrial grafts as demonstrated by Markee (1929), and the second was based upon the collection and extraction of excrement of the animals, with tests

for oestrin being conducted by injection of the extracts into spayed female rats. Each method gave suggestive indications but each was far from satisfactory.

Endometrial grafts.—Markee (1929) demonstrated that grafts of small pieces of the uterine mucosa in the anterior chamber of the eye underwent regular changes of vascularity during the oestrous cycle. During dioestrus the mucosa alternately blushed (became more vascular) and blanched, the complete vascular cycle requiring from twelve to twenty-five seconds. During oestrum the graft showed a continuous blush reaction. Markee further showed that ovariotomy abolished the rhythmical changes and led to a continuous "blanch" with disappearance of all "blushes," and that injections of "menformon" (oestrin) returned the continuous blush. Alternate blush and blanch therefore is strong indication of the dioestrous phase of the cycle, whereas continuous blush indicates oestrum.

As a means of attempting to determine whether ovarian grafts were cyclic, small pieces of uterine mucosa were transplanted into the anterior chamber of the eye of male guinea pigs castrated some months earlier, and at the same time an ovary was transplanted into the kidney. Several successful mucosa grafts were obtained in animals with functional ovarian grafts as indicated by mammary responses, but a number of these were complicated and rendered useless by the fact that the cornea became opaque shortly after observations were begun.

Five animals were obtained in which functional ovarian grafts were present and which had successful endometrial grafts, but none of the 5 could be followed for more than a few weeks time. Examination involved daily observations upon the state of the endometrial graft. These 5 animals exhibited a definite blush reaction for several days at a time but there were periods in which distinct blush and blanch periods alternated.

The difficulties involved in this attempt are enormous and the data fragmentary and admittedly lacking in the conclusiveness desired, but so far as they go they give strong indication that the ovarian graft does not maintain a continuous rate of secretion but varies from time to time in the rate of oestrin secreted. This is, of course, the essence of a periodicity that is characteristic of the

ovary in a female animal. It tells nothing concerning the regularity of the process or its degree of approach to the cycle characteristic of the normal female.

Oestrin excretion.—It is known that, in some animals at least, oestrin can be collected from the excrement, particularly the urine of pregnant females. After well-known extraction methods oestrin is easily detected by injection into spayed female rats; cornified smears from the vagina give a positive indication of the presence of oestrin. To determine whether this method possessed any merit for the guinea pig, the total excrement (urine and feces) from a number of pregnant females was collected, dried, and extracted; and, when suspended in olive oil, was injected into spayed rats. The cornified smears proved that it was possible to detect oestrin by such relatively crude technique. With this encouragement an attempt was made to determine whether oestrin elimination was periodic in males bearing functional ovarian grafts.

The total excrement from each of 6 guinea pigs was collected for periods of three days followed by a period of three days without collection. This was made necessary by the fact that the animals were confined in small wire meshed cages suspended above earthenware bowls, and continuous residence in such quarters led to body weight losses and a general decline in good health. The animals occupied the small cages for approximately twenty hours each day, as they were fed in other cages for a period of four hours daily. Each three days of collection, therefore, was followed by a three-day period of rest during which the animals were kept in larger cages with a continuous supply of food. The total excrement from each individual animal for each three days of collection was dried, weighed, powdered, and extracted with benzene continuously for ten hours in a Soxhlet extractor. The benzene was distilled off and the temperature of the residue raised to 110° C. to rid it of benzene and water. The tar-like remains were dissolved in ether and 10 cubic centimeters of olive oil were added to the solution. After evaporation of the ether the material remained in olive oil solution. For assay, $\frac{1}{2}$ cubic centimeter of the oil solution was injected daily for three days into 4 or 5 spayed female rats and vaginal smears were taken twice daily. The 6 guinea pigs from which the excrement was so treated consisted of 2 normal

females, 3 castrated males bearing functional ovarian grafts, and 1 experimental cryptorchid male bearing a functional intra-renal ovarian graft.

TABLE III

THE OCCURRENCE OF OESTROGENIC SUBSTANCES IN THE EXCRETA OF OVARIAN-GRAFT-BEARING ANIMALS

Experimental Cryptorchid Males			Castrate Male	Normal Females			
468	449	407	222	A		B	
				vaginal smears		vaginal smears	
+	+	+	−	oestrum	+	oestrum	+
+	−	+	+		−		−
−	−	−	−		−		−
				oestrum		oestrum	
−	−	−	−		−		−
−	−	+	+		−		−
−	−	+?	−	oestrum	−	oestrum	+
+	−	−	+		−		−

Excreta (urine and feces) collected on alternate 3-day periods. Blank spaces represent the periods during which no collection was made. Plus (+) indicates the presence of oestrogenic substances in that sample as shown by the positive reaction of all of the assay animals. Minus (−) refers to a negative reaction of all of the test animals on injection of excrement extracts into spayed females.

The results obtained from an assay of the excrement of this group of 6 animals is given in brief form in Table III. The method proved sufficiently delicate to detect oestrin in the excrement from the normal females during the period of oestrum but gave no indication of its presence during the dioestrous period; these periods were of

course easily determined in the normal females by the vaginal smears. The excrement collected from the males bearing functional ovarian grafts was positive during some of the three-day periods but negative during others. In some cases positive reactions were given for two succeeding three-day periods.

These observations are interpreted to mean that the ovarian grafts in the castrated and experimental cryptorchid males were producing sufficient oestrin to be detected by injecting the extracts from the total excrement into spayed female rats. The amount of oestrin excreted within a period of three days was not constant but varied between such extremes as easily detectable amounts and amounts that were not detectable by the methods employed. The data obtained do not serve to indicate the regularity of this cyclic event nor the total time between two most active periods of excretion, because instead of showing results for a continuous period sufficiently long to encompass a normal cycle of the female it covers only periods of three days separated by other three-day periods for which no data are available.

Both the endometrial graft observations and those made from the collection of excrement, therefore, indicate very strongly that the period of oestrin secretion by these ovarian grafts in treated males varies rather than remains constant. The fact that such periods of more abundant secretion occur is good evidence that cyclic phenomena characterize the activity of these grafts, but it tells us little concerning the regularity of these periods or how nearly comparable they are to the similar phenomenon that is the regular occurrence in the normal ovary of the female. The observations do not support the suggestions of Lipschütz (1927*a*) that cyclic phenomena are absent in ovarian grafts in males, hence some other explanation must apparently be adopted to account for the difference in mammary responses to ovarian grafts in spayed females and castrated males.

HYPOPHYSIAL DIMORPHISM

The difference between the responses of the mammary apparatus in the castrated male and the spayed female guinea pig in which ovarian intra-renal grafts are the responsible agents would seem to have been clearly demonstrated, by the observations recorded in

preceding portions of this discussion, to be dependent upon the amounts of oestrin liberated by the grafts. The reactivity of the male and female mammary glands to injections of oestrin was shown to be essentially identical. The key to the difference therefore rests with those forces which govern the output of oestrin from the grafts. The suggestion of Lipschütz (1926, 1927*a*), that the difference depends upon the lack of periodicity in the ovarian graft in the male does not receive substantiation from the material which is here reported.

The most plausible explanation that suggests itself involves the well-known stimulant to gonad activity—the hypophysis. This gland which controls the activity of the ovary must therefore exhibit a physiological sex-dimorphism which is responsible for the greater oestrin production of ovarian grafts in the male than in the female organism. That such a physiological sex-dimorphism is operative under normal conditions is indicated by the fact that hypophyses from male animals are more potent in stimulation of the gonads of prepubertal animals than are those of females (Evans and Simpson, 1929). Is this a condition imposed upon the male hypophysis by secretions from the testis and a lower capacity imposed upon the female hypophysis through secretions from the ovary, or is it a genetic character arising without any relation to secretions from the gonads as the animal develops to maturity? If it is a condition—a physiological reaction—that is in any way conditioned by the development of the respective sex hormones, early gonadectomies accompanied by ovarian transplantation should lead to similar responses on the part of the mammary apparatus in both sexes. If, on the other hand, it is a genetic character the same differences in mammary response in the two sexes should follow, even after very early gonadectomies, as followed transplantations in adult animals. To learn the relative responses in the young animals a group of brother-sister animals was utilized.

Four pairs of brother-sister guinea pigs were selected and the male was castrated a few days after birth, receiving at the same time one ovary from its sister as an intra-renal graft. The male and female thus possessed one ovary each, but differed in that the ovary in the male was a graft, whereas that of the female was normal (had

not been disturbed). Measurements of the nipples were recorded for a period of six months, and the trend in nipple growth is recorded in Figure 9.

At the termination of the experiment the nipple height of all the treated males was considerably greater than the nipple height of their sisters carrying the corresponding but undisturbed ovary.

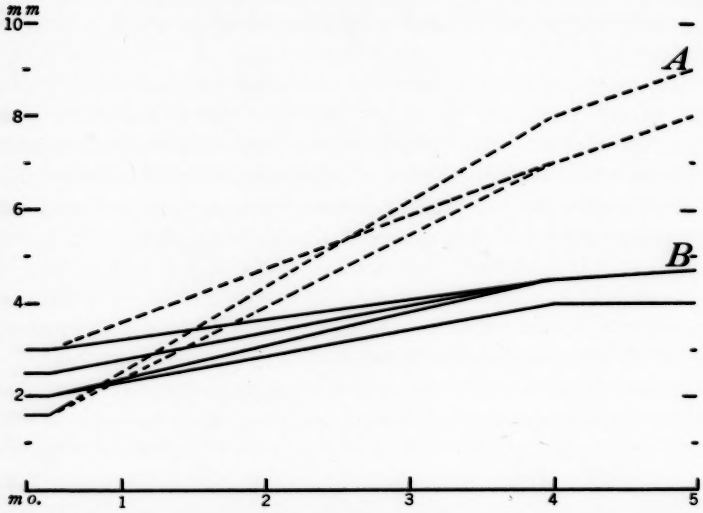

FIG. 9.—Growth response of the nipples of young prepubertally castrated males (*A*) to ovarian grafts made before puberty. The controls were unilaterally spayed sisters (*B*) of the males, and donors of their grafts. The months refer to the age of the animals.

Thus the nipple height of the males was from 8 to 9 millimeters whereas the nipple height of the females was from 4 to 5 millimeters.

In order to eliminate a factor which might have affected the result due to the undisturbed ovary of the female, in contrast to the grafted ovary of the male, a second set of animals was utilized in which each sex carried only a grafted ovary. Five pairs of brother-sister animals were utilized in which the male was castrated before the age of fifteen days and received at the same time one ovary from its sister. At the same time also the second ovary was removed from its normal site and placed in the kidney of the female donor (auto-

graft). The 2 animals were therefore equal in every respect with the exception of their genetic constitution.

The growth of the nipples of these two groups of animals is expressed in Figure 10. Nipple growth began about the normal age of puberty and in some cases was apparent considerably earlier in the male than in the female. Furthermore, the growth of the male nipples was more rapid than that of the females and when observations were discontinued were much larger than those of the females.

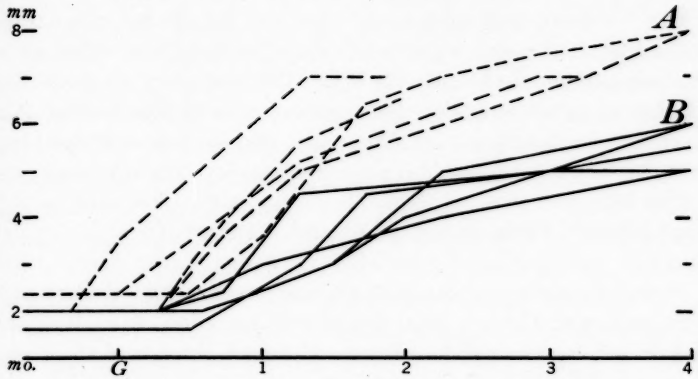

FIG. 10.—Growth response of the nipples of young prepubertally gonadectomized males (*A*) and females (*B*) to ovarian grafts made before puberty. The months refer to the age of the animals.

This result appears to be open to but one interpretation—namely, since the two ovaries from the female donor were transplanted at the same time, in the same manner, and to similar positions, their inherent potentialities should have been equal. Differences that they may exhibit must lie outside their own domain and be conditioned by those forces which become operative upon them. Since the hypophysis is known to govern the activity of the ovary and control its oestrin production, and since it has been shown earlier in this paper that the potential reactivity of the male and female mammary glands to oestrin is the same, the greater oestrin secretion by the graft in the male must be attributed to a greater stimulation issuing from the hypophysis. All influences emanating from the sex glands

were removed before they could have become sufficiently potent to condition the responses of other organs of the animal, such as the hypophysis. The explanation must rest, therefore, upon a physiological sexual-dimorphism exhibited by the hypophyses, with the capabilities for ovarian stimulation being greater in the hypophysis of the male than in the female. It would therefore seem to be a sex-limited genetic character.

LACTATION

Among the large group of treated males bearing active ovarian grafts, 2 animals only gave any evidence of lactation. One of these was a castrated male, the other an experimental cryptorchid male. In each case nipple height was below the maximum attained in a number of other animals. The amount of milk expressible at a given time consisted of a few drops only, and the flow continued but for a few days. The reason for the discrepancy between results reported here and those of Steinach (1913, 1916), Lipschütz (1926), Sand (1922*c*), Athias (1915, 1916) and Pettinari (1925) is not yet clear.

It was thought possible that for some reason male guinea pigs with ovarian grafts were incapable of milk secretion. A study of the ovarian grafts revealed the absence of corpora lutea, and the mammary gland tissue in sections resembled that of a female in an early stage of pregnancy rather than one near parturition. Since many authors have ascribed the development of a functional mammary gland to the corpus luteum and since it is well known that administration of anterior pituitary extracts induces corpus luteum formation and stimulates milk secretion in the adult female, the effects of injection of such substances into the graft-bearing males were investigated.

Three castrated males and 3 experimental cryptorchid males, each bearing a functional ovarian graft, were injected twice daily for a few days with an aqueous pituitary extract (an alkaline extract of sheep pituitary prepared by Dr. O. Kamm of Parke Davis Co.). Whereas there was no measurable increase in the nipple height from this treatment, these males secreted milk (preceded by colostrum) within fifty hours from the time of the first injection.

When the sections of the ovarian grafts from these pituitary ex-

tract injected males were studied, well-developed corpora lutea were found.

In order to determine whether the presence of a corpus luteum in the ovarian graft was required for lactation, 3 treated male animals with large nipples were injected with the aqueous pituitary extract eight hours after removal of the kidneys in which the ovarian graft was located.

Despite the absence of any definite corpus luteum tissue in the graft that was removed before the pituitary extract injection, these males all lactated as in the cases previously cited.

In order to eliminate all possible sources of corpus luteum secretion, as from diffuse tissue possibly giving secretions equivalent to a well-formed corpus, experimental cryptorchid males which had not received ovarian implants were utilized. These males, with testes confined in the abdomen, were injected with oestrin known to be free from the lutein hormones until their nipple development was as great as that of a pregnant female (a height of 9–11 millimeters). The animals were then injected with the pituitary extract, and milk secretion began within forty-eight hours.

The results of this group of experiments clearly demonstrate that the hypertrophied mammary apparatus of the treated male guinea pig is capable of lactation if only anterior pituitary substances are administered.[3] They demonstrate further that lactation can be induced in the male mammary glands without lutein secretions ever having been involved in the stimulation. The results, however, do not indicate that the corpus luteum secretions are useless in ordinary lactation in normal females.

The absence of lactation in practically all males bearing functional ovarian grafts and well-developed mammae may be due to the lack of anterior pituitary secretions, the production of which is inhibited by oestrin produced by the graft. The degeneration of such a graft followed by a decrease in oestrin secretion would cause a sudden increase in anterior pituitary hormone available, which when present in sufficient quantities has been shown to result in lactation in such animals. An analysis of this suggestion is in progress.

[3] This question will be treated at greater length in a subsequent publication in conjunction with Dr. W. O. Nelson.

EFFECT OF OVARIAN GRAFTS ON TESTIS HORMONE SECRETION

The enormous growth of the nipples of both the castrated males and the experimental cryptorchid males guarantees that relatively large amounts of oestrin were being produced by the ovarian grafts. Several writers who have injected the normal male with oestrin have reported an "anti-masculine" effect of some character, involving such structures as the gonads and accessory reproductive organs (see Laqueur, Borchardt, Dingemasse, and De Jongh, 1928; Steinach and Kun, 1926 and 1931; Fellner, 1921; Moore, 1930*a;* and Moore and Price, 1932). Some of these authors have attributed the result to an antagonism between the sex hormones, but Moore and Price have assembled a great weight of evidence pointing to a reciprocal influence between the sex glands and the hypophysis as the probable explanation. Thus, they maintain that introduction of oestrin, rather than having a direct effect upon the testes and accessory organs, acts to interfere with the hypophysis in such a manner that the hypophysial secretions necessary to maintain gonad function are diminished, and the injury to the male system would thus be due only to a deficient amount of these necessary substances. If this explanation is correct, one would expect that the presence of the oestrin secreted by the ovarian grafts in experimental cryptorchid males would in some way modify the secretion of testis hormones by the abdominal testes.

In order to determine whether there was evidence for diminished testis hormone secretion, a study was made of the amount of the ejaculate each week as determined by the electric ejaculation test (see Moore and Gallagher, 1930).

The ejaculate was obtained each week from a group of 21 experimental cryptorchid males carrying functional ovarian grafts. These ejaculates were compared with those collected from a group of 18 normal males. They were further compared with ejaculates from a group of 27 experimental cryptorchid males, 19 of which had received ovarian transplants that failed to become incorporated, and 8 of which had never received ovarian transplants. The record of these observations is given in Figure 11. The first ejaculate obtained from each animal was discarded in order that those considered

might more adequately express the rate of secretion on the part of the prostate and seminal vesicles which are the principal source of the material composing the ejaculate. The first ejaculate rather than indicating the amount of secretion for the previous week more often is larger in amount from the factor of storage of the secretions from several days longer than one week. The average of all ejaculates from each animal collected, subsequent to the first, would therefore

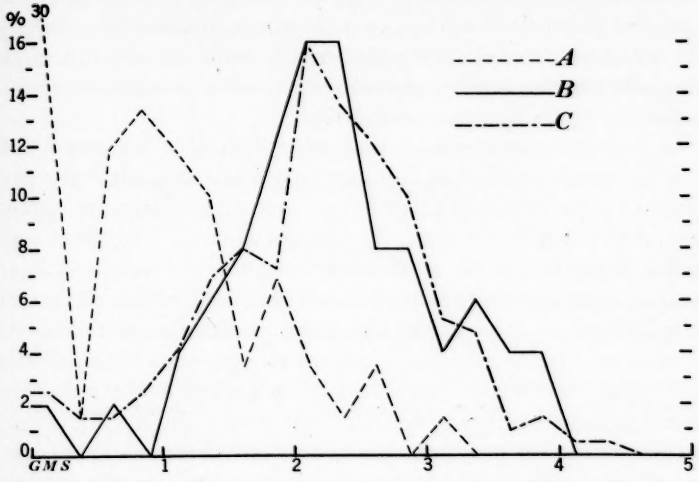

FIG. 11.—Distribution curves of the ejaculate weights of: *A*, cryptorchid males bearing ovarian grafts (59 weights); *B*, normal males (50 weights); *C*, cryptorchid males (191 weights).

establish quite satisfactorily the average rate of secretion of the accessory organs. This would in turn serve as an index of the rate of testis hormone secretion, since the activity of these organs is under the direct control of the testis.

Figure 11 shows the ejaculates plotted in a distribution curve for the three groups. The curve of the ejaculate distribution from the ovarian-grafted cryptorchid males is based upon 191 points. The curves for both the normal males and the experimental cryptorchid males are almost identical, which shows that the abdominal testes entirely devoid of germinal tissue are secreting as much testis hor-

mone as do the testes of the normal males. The secretion of that portion of the testis hormone, therefore, which controls the accessory reproductive organs remains unchanged in the degenerate abdominal testicle. On the other hand, the amount of ejaculate produced by the experimental cryptorchid males bearing functional ovarian grafts is much below that of the control groups. This is interpreted to mean that the oestrin secretion of the ovarian graft serves effectively to inhibit the hypophysis in its activity and to reduce the quantity of hypophysial secretions available to stimulate the secretory activity of the abdominal testes. As a result of the diminished testis hormone production a smaller amount of ejaculate is obtained after electrical stimulation.

In order to determine more absolutely, if possible, whether oestrin does have such an effect in the male guinea pig, 16 adult males were rendered experimentally cryptorchid, were divided into two groups of 8, and ejaculations were collected each week for a period of eight weeks. Beginning at the ninth week one group received daily injections of 1 rat unit of oestrin for a period of four weeks; the second group served as the control. The daily injection of oestrin in the experimental group was then increased to 2 rat units for a period of two weeks, then to 4 rat units for two weeks, and finally a daily injection of $12\frac{1}{2}$ rat units was given for four weeks. The average weight of the ejaculates from all animals of each group is plotted in Figure 12 and covers the eight weeks preceding injection and the consecutive weeks during which the different dosages of oestrin were administered.

An examination of this graph reveals the fact that the ejaculate curve from the group of animals receiving a daily injection of 1 rat unit of oestrin diverges from that of the uninjected ones to a detectable degree. As the dosage was increased to 2, 4, and 12 rat units daily, the average weight of the ejaculate of the injected group decreased, whereas that of the control group remained essentially constant. This result shows that oestrin injection does cause a decrease in the secretory activity of the accessory reproductive organs, which in turn is to be explained only on the basis of a lowered hormone secretion by the cryptorchid testes. This lowered testis secretion is to be explained on a diminished available amount of hypophysial

secretions caused by the activity of oestrin, which is injurious to the hypophysis. The results, therefore, confirm the contentions of Moore and Price that a reciprocal physiological influence is operative between the gonads and the hypophysis. Their data which formed the basis for such an explanation were obtained almost entirely from the rat, and my own observations on the guinea pig offer strong confirmative evidence that the explanation is the correct one.

The conclusions drawn from material in this section are that an ovarian graft causes a diminution in the function of the accessory organs of the male guinea pig, and that the influence is probably due to the secretion of oestrin. Purified oestrin samples given in sub-

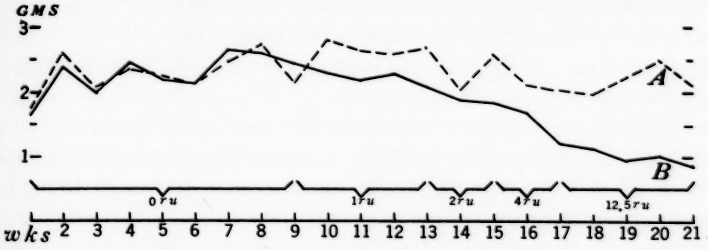

FIG. 12.—Average ejaculate weight of experimental cryptorchid males. *A*, controls (8 animals) uninjected. *B*, treated males (8 animals)—no injection for first eight weeks after testis elevation. Succeeding four weeks one rat unit of oestrin injected daily; succeeding two weeks 2 rat units of oestrin injected daily; final four weeks 12.5 units of oestrin daily.

cutaneous dosages of known strength were found to have an influence entirely similar to that exerted by the functional grafts. The diminished secretion of these accessory structures is due to a diminished secretion of testis hormone. The manner in which oestrin causes a lowered testis hormone production involves an interference with hypophysial function.

BEHAVIOR OF OVARIAN GRAFTED MALES

One of the many interesting phases of the problem of the effect of sex hormones upon the organism is that reputed to affect, or condition, behavior. A systematic and careful study of this phase has not been attempted in this investigation, but in numerous instances casual observations made on the group of animals with which I have

worked lead me to offer them only as observations without attempting a careful analysis or extensive interpretation.

Normal males kept in groups of a dozen or more in large pens fight considerably. It is also true that females will fight among themselves but not to the extent exhibited by males. Experimental cryptorchid males behave entirely as normal males. Prepubertally gonadectomized males and females seldom fight. Pregnant females, or those suckling young, drive away other animals of both sexes. Males may mount one another in an attempted coitus and may often experience an ejaculation, but a penchant of one female for another has not been noticed.

Experimental cryptorchid males bearing functional ovarian grafts, as testified by enlarged mammae, fight among themselves and mount in typical male fashion. Their mating reactions with females are as typical as those of normal males.

In the course of the observations on lactation, young suckling animals a few days after birth were taken from their mothers and confined with the lactating males in small pens. Although driven by hunger to make repeated and vigorous attempts to suck, these young animals were consistently and vigorously repelled by the male, castrated a few days after birth and carrying a functional ovarian graft for months. The animal was lactating, and, when it was forcibly held, the young did nurse. However, not even passive resignation was exhibited after forceful restraint for several days during which the young nursed. Among the large number of castrated males with active ovarian grafts that have served as a basis for this study, none has exhibited any tendencies that could be interpreted as showing the possession of maternal instincts. The behavior reactions of these male animals were entirely characteristic of males though they had lacked testes since before puberty. Such observations are in disagreement with those of Steinach (1916) and Sand (1918); they substantiate similar observations of Moore (1921*b*).

HISTOLOGICAL CHARACTER OF OVARIAN GRAFTS

A remarkable agreement appears to exist in the several accounts of the structure of ovarian grafts in male animals. In general one finds considerable follicular atresia, a tendency toward cyst forma-

tion, and an absence of well-defined corpora lutea. Voss (1926) notes a striking tendency toward calcification in the connective tissue.

The ovarian grafts examined by me have exhibited practically the entire range of possibilities described by others. The simple technique of Bouin fixation and haematoxylin and eosin staining has been employed, and all cases involved the tissue surrounding the graft—either kidney or testis tissue—depending upon the locus of the graft. The implantation sites of 75 positive cases and 50 negative cases (those in which mammary responses were absent) have been sectioned serially. Since the reactions of grafted ovaries have been treated so often in previous writings, and since my own observations introduce no particularly striking new facts, a brief mention only of the conditions found will be attempted.

The one outstanding difference in ovarian grafts recovered from males (castrated or experimental cryptorchids) and those recovered from females is the presence of well-formed corpora lutea in the latter group. As mentioned in a previous section, those males receiving injections of pituitary extract produced typical corpora. But in addition to this outstanding difference it appears to be a general rule that grafts from the males contained more numerous and larger Graafian follicles than did those from females. The number of small (primary) follicles, as a rule, was not great, but this may not be surprising in view of the fact that a germinal epithelium was absent in all cases, due to the mode of incorporation of the graft in the host tissue. Furthermore, most of the ovarian grafts removed for section had persisted in the hosts for periods ranging usually from twelve to eighteen months.

All grafts recovered from animals that had given positive mammary response contained at least a few normal follicles, whereas no graft studied from a case in which mammary response was negative contained normal follicles.

Ovarian grafts recovered from experimental cryptorchid males exhibited no marked differences from those recovered from castrated males. Each group revealed normal follicles in different stages of maturity that contained normal-appearing ovocytes. The fact that the experimental cryptorchid males were producing testis hormone in amounts equivalent to that produced in normal males and yet

showed no modification in the response of the grafted ovary is important.

It follows from these observations, therefore, that the normal secretion of testis hormone in experimental cryptorchid male animals is without demonstrable effect upon the reactions of the ovarian tissue in either an anatomical or a physiological sense.

DISCUSSION

The problems involved in the general question of sex hormone antagonism as it has been introduced by Steinach, modified by Sand, Lipschütz, and others, and criticized by Moore, and Moore and Price, are broad and complex.

It is no longer to be doubted that the original contentions of Steinach, that heterosexual gonad grafts could not be successfully made in a normal host of the opposite sex, were incorrect. This has been disproved adequately by the publications of Sand, of Lipschütz and his co-workers and students, and of Moore, and is further disproved by the observations presented here and by many casual observations of other workers. Certain of the contentions in Sand's modification of Steinach's antagonism conception are wrong, but others, at least with certain modifications, will stand. Thus Sand's failure to obtain persistence of an ovarian graft in a normal male host in localities other than the testis is not only unimpressive but thoroughly misleading in its implications. Lipschütz and his students have demonstrated many times that ovaries transplanted into the kidney of a normal male will become incorporated successfully and persist for a long period of time. This is adequately confirmed by the material herein presented. That such ovarian grafts seldom exercise their potential hormone-secreting capacities is another question and will be dealt with below. The testicle, therefore, does not present any particular merits as a locus for ovarian grafts excepting for its accessibility, size, and excellent vascular bed. Transplantation of ovaries into the kidney or other sites is just as successful as transplantation into the testicle.

The contentions of Sand, involved in his familiar "atreptical immunity" hypothesis (1918, 1919, 1922*a*, *b*, *c*, and 1923), when changed to fit more recent contributions to our knowledge regarding

sex gland activity has considerable merit. Persistence and function, rather than being dependent upon the capture by the testis of certain "food substances in the circulating blood stream" thus making them available to an intra-testicular ovarian graft, can be understood to mean the dependence of the ovarian grafts upon certain substances produced by the hypophysis for their persistence and function. Not only does this idea stand confirmed, but it indicates one of the first signs of recognition of the dependence of the gonads for persistence and function upon substances produced elsewhere in the organism. Instead of a nutritive principle in the generally accepted sense, however, it must be recognized as a hormone stimulation, the secretions responsible for the action coming from the hypophysis.

A further confusing contention in the general problems involved is that sex hormones produced in the two sexes are antagonistic, but the degree or character of such an antagonism has not been well defined. Steinach's earlier conception of the direct inhibition or antagonistic effect of a hormone upon the heterologous end organs is no longer tenable if due respect is paid to the observations presented by Moore and Price, and to those contributed in this publication. Thus, Moore and Price have adequately demonstrated that the accessory reproductive organs of the castrated rat will respond to a given dosage of testis hormone in like manner when this is administered alone, or accompanied by large potent dosages of oestrin. Similarly, the mammary gland of the guinea pig will respond to minimal effective doses of oestrin either in the presence, or absence, of large quantities of testis hormone.

There are other difficulties and somewhat confusing conditions, however, for, as Lipschütz has demonstrated and the present work confirms, the presence of a normal testicle, and of an abdominal testicle that is devoid of germinal epithelium, exerts different effects upon the activity of an ovarian graft. The normal testicle, though permitting the successful incorporation and persistence of an ovarian graft, will usually prevent it from carrying on its endocrine secretion. The abdominal testicle which lacks an active germinal epithelium, however, not only permits the incorporation of an ovarian graft, but also permits as great an endocrine secretion by

the graft as ocurs in a castrated male. Each of these two types of testes are accompanied by the same degree of activity on the part of the male accessory reproductive organs—seminal vesicle, prostate gland, etc.—hence, in the usual sense, they can be said to secrete equivalent amounts of testis hormone. This introduces the question of "multiplicity" of the testis hormone. Is the internal secretion of the testicle one substance, or does it consist of more than one substance?

The testis hormone, entire or in part, has been extracted from the fresh testicle of the bull and other mammals, and from human male urine (McGee, 1921; Gallagher and Koch, 1929; Funk, Harrow, and Lejwa, 1930; Loewe and Voss, 1929; Freud, de Fremery, and Laqueur, 1931), and a crystalline product from urine has been obtained by Butenandt (1931). The chief methods of assay have utilized the capon comb method, and whereas the material stimulates the capon comb growth without question, it is still to be demonstrated whether such substances as have this power include all the substances that are normally secreted by the mammalian testis.

In a co-operative study of the testis hormone in this University, mammals have been utilized extensively in testing the effectiveness of the substances prepared by Gallagher and Koch from bull testis extract. The reactions in the mammal to these substances have been studied by Moore and his co-workers. They have attempted an exhaustive study of the effects of castration in rats and guinea pigs particularly, in order to determine as nearly as possible all the effects that follow complete elimination of the testis. As a result of these studies approximately nine or ten hormonal deficiency effects have been described which encompass a variety of hormonal functions. These have included the effects upon the life of unshed epididymal spermatozoa in the guinea pig (spermatozoan motility test) and the physiological activities of the accessory reproductive organs as determined by inducing an ejaculation in the guinea pig by electrical stimulation on the head (electric ejaculation test). They have included also a thorough investigation of the accessory reproductive organs in the rat—seminal vesicles, prostate gland, Cowper's gland, vas deferens, epididymis, etc.—by cytological methods.

Moore has repeatedly stressed the necessity of learning all the deficiency effects following castration before concluding finally that a given preparation of hormone contains all the substances normally secreted into the blood stream by the testis.

The indication that the preparations of Gallagher and Koch contained the complete hormone or hormones was given by the fact that their injection into castrated animals served to ward off all detectable effects following testis ablation.

An effect of castration more recently being emphasized, and not yet entirely clear, is the response of the hypophysis both anatomically and physiologically. It was shown by Engle (1929), Evans and Simpson (1929), and others that castration is followed by an increased potency of the hypophysis to stimulate sex gland activities upon its implantation into immature animals, and it has been noted by many writers that cellular changes in the hypophysis accompany such an increase in stimulating powers, especially in the rat ("castration cells"). There is some evidence that these hypophysial responses to testis hormone, or its absence, depend upon substances not involved in the regulation of the accessory organs. Thus Martins and Rocha (1931*b*) claim that certain extracts from the testicle will prevent the development of castration changes in the hypophysis of the rat and yet be incapable of exercising a hormonal control over the accessory organs. Still other extracts that adequately maintain the normal condition of the accessory organs do not prevent the appearance of castration cells. Furthermore, experimental cryptorchid animals possessing entirely normal accessory reproductive organs show the typical castration cells. The substance having the regulatory control over the hypophysis is thus believed to have its source of origin in the germinal epithelium, whereas it is well known that the accessory reproductive organs are maintained indefinitely in a normal state in the complete absence of a germinal epithelium (experimental cryptorchid guinea pigs).

The work of Lipschütz, and that reported here, clearly demonstrates a different response on the part of an ovarian graft in the presence of testes with an active germinal epithelium and those without it. But it is not entirely clear from this and other work whether the differences do not involve a quantitative factor in the

reciprocal gonad-hypophysial interrelationship. If there are two separate components to the testis hormone, one exercising a control over the hypophysis, the other over the accessory reproductive organs, it would appear that they are obtained by the same methods of extraction and purification. The preparations of Koch and Gallagher have been shown to be entirely adequate in maintaining the normal conditions of the accessory reproductive organs in the castrate mammal (Moore and Gallagher, 1930; Moore, Hughes, and Gallagher, 1930; Moore, Price, and Gallagher, 1930; Vatna, 1930; and Heller, 1932); and, after the injections of Gallagher's preparations, and others prepared in a similar fashion, into castrate rats, Reese and McQueen-Williams (1932) claim that castration cells are also abolished. These results indicate either that a single substance is involved in the hypophysial and accessory reproductive gland control or that if more than one is involved all are present in these extracts.

Assuming therefore that the complete testis hormone is contained in the testis extract preparations of Gallagher and Koch, one must immediately grant the contention that no antagonism exists between these and oestrin. The delicate response of the mammary glands of the guinea pig, as herein described, to amounts of oestrin of the order of two-tenths of a rat unit daily is clearly demonstrated. When this quantity is administered, along with sufficient quantities of testis hormone to produce coagulable ejaculates in the castrated males, and yet produces the same response in the mammary glands as when injected alone, there appears to be no cause for considering an antagonism to exist between the two substances.

Such basic information in regard to mammary response, and this applies to either male or female mammary glands, permits an approach to consideration of the response of these end organs to ovarian transplantation.

In the normal male guinea pig ovarian grafts persist but only seldom do they evoke a response in the mammary glands. This is not due to the presence of the testicle or its secretions, except indirectly, but only to the fact that the graft is not secreting hormones; for, when oestrin is injected into the normal male, mammary response follows. It is obvious, therefore, that the ovarian graft in the normal

male does not secrete oestrin. That it is capable of so doing is easily demonstrated either by removal of the testicle or by elevating it into the abdomen. It appears reasonable to believe that the lack of oestrin secretion is due merely to the insufficient quantity (possibly quality?) of those substances that force an ovary to secrete—namely, hypophysial sex-stimulating substances. This hypophysial sex-stimulating substance can be increased in a male in at least three ways: (1) It is increased by removal of the testis. This is conceived to act in two possible ways. In the first place, the testicle probably utilizes the substance in some manner in its general metabolism. This is indicated by the fact that in administration of the sex-stimulating substance the response is at least roughly proportional to the amount administered; nothing is known, however, in regard to its mode of action. In the second place, castration removes the suppressing influence of the sex hormones on the hypophysis. That the hypophysis is suppressed by the sex hormones and is more active in their absence is proved by gonadectomy (Engle, 1929; Evans and Simpson, 1929) and by injection of the sex hormones (Meyer, Leonard, Hisaw, and Martin, 1930; Moore and Price, 1932; Spencer, Gustavson, and D'Amour, 1931; Wade and Doisy, 1931). (2) The hypophysial sex-stimulating substance can also be increased by rendering the animal an experimental cryptorchid. Hypophyses of experimental cryptorchid males are intermediate in their activity between those from normal males and the more active ones from castrated males (Evans and Simpson, 1929; Kallas, 1930; Martins and Rocha, 1931). (3) Finally, the sex-stimulating substance can be increased in the animal by direct injection. The first two methods lead to an immediate response of the mammary glands in the guinea pig bearing an ovarian graft, as shown by both Lipschütz and myself. The third method, that of direct injection, has also been shown by Engle (1928), in the male rat bearing an ovarian graft, to induce mammary hypertrophy while the normal testes were present and active. In the rat it was the mammary gland tissue which responded and not the nipple, since male rats do not possess rudimentary nipples.

It can be readily understood, therefore, that the conditions which prevent the ovarian graft from exercising its potential influence upon

the mammary gland of the normal male are believed to be due merely to the fact that the hypophysial substances are not available to stimulate ovarian graft activity because of the activity of the testicle. There is a sufficient amount of secretion for the activity of the testes but not for both testes and ovary. It is not clear why the testis can maintain its activity and the ovary only incompletely so. Threshold differences of the ovarian and testicular tissues might be a factor, and it is also conceivable that qualitatively different substances are involved. The presence of the testicle is antagonistic but only by virtue of its influence upon the hypophysis, and not from any inactivation of oestrin or any inhibitory influence upon the mammary gland.

It is an interesting fact that the mammary gland responses to ovarian grafts in castrated males and experimental cryptorchid males are greater than those in spayed females with ovarian grafts or in those of the normal female. It must be remembered that this difference is not due to a difference in the capacity of the mammary glands of the two sexes to respond to oestrin. Their equipotentiality in the two sexes is clearly demonstrated by the experiments here reported. The difference depends here again entirely upon the actual secretion of oestrin. The fundamental question, therefore, is why ovarian tissue in the male soma secretes more actively than that in the female soma. Again the answer appears to be obscured by lack of knowledge concerning the function of the hypophysis and the interactions between it and the sex hormones. Ovaries respond to hypophysial secretions and produce more oestrin but appear to be modified in their response by the formation of corpora lutea. There is general agreement that corpora lutea are formed in ovarian grafts in the female but not in the male. Is this due to quantitative differences in the male and female hypophysial secretions or to qualitative differences? It is impossible at the present time to give the final answer, but the investigations of many workers and observations of my own make it seem highly probable that a genetic difference exists in the hypophysis of male and female animals. Hypophyses of normal males are more potent in stimulating precocious sexual maturity than those of normal females; and those of castrate males are more potent than those of spayed females (Evans and

Simpson, 1929). Furthermore, as my experiments show, of two ovaries from the same immature female transplanted, one into the kidney of a castrated brother, the other into her own kidney, at the age of ten days (therefore each animal was without gonads except the ovary graft), the graft in the male soma invariably secreted more oestrin than the one in the female soma. This fact, however, does not demonstrate whether the cause lies in a quantitative or qualitative difference in the two hypophyses. The gonad-hypophysial interactions are complicated in the female, not only by the apparent quantitative difference (perhaps qualitative) in its secretory activity, but also by the formation of lutein tissue. Secretions from the latter appear, from the work of others, to influence the response of the ovarian tissue and in the second case they may also affect the activity of the hypophysis in a manner different from that of oestrin. The solution of the problem of the differences in ovarian response in the male and female soma must await further study.

The question of the periodicity in function of ovarian grafts in the male is not satisfactorily disposed of by my observations. Periodicity in ovarian function in the female is an outstanding characteristic. The mechanism involved in the regulation of this periodicity as explained by Moore, 1930*a;* Brouha and Simmonet, 1930; Moore and Price, 1932, are the reciprocal interactions between the ovary and hypophysis. Thus Moore, and Moore and Price visualize the cycle, starting with the ovary, as first dependent upon a stimulation of the ovary by secretions from the hypophysis. This leads to follicular ripening and an outpouring of oestrin. Oestrin then brings on the oestral response (vaginal cornified smears and mating), and the concentration of oestrin serves effectively to arrest hypophysial secretion. The hypophysis therefore exhibits a low state of secretion during the time oestrin exists in a high concentration in the body, and it is during this time that the ovary is inactive in follicular development. When the oestrin concentration is reduced in the body, through excretion, the hypophysial activity occurs, thus inducing a new cycle. It is well understood, of course, that many conditions can operate to disturb this delicate controlling mechanism and lead to irregularities or total suppression of the cycle.

Granting for the moment the possible correctness of the explana-

tion of the mechanism, it does not follow that the male hypophysis would fit into the scheme as adequately as does the female hypophysis. The discussion above takes into consideration some of the evidence that the hypophyses of the two sexes are fundamentally different in some phases of their physiology. That the male hypophysis is affected by oestrin is clearly indicated by the work of Moore and Price, 1932; Spencer, Gustavson, and D'Amour, 1931; and Wade and Doisy, 1931; but these experiments do not prove that the threshold of effect is the same as that for the female hypophysis.

The evidence presented in this paper on the activity of endometrial grafts in the anterior chamber of the eye of males bearing ovarian grafts, and that from the excretion of oestrin by males with ovarian grafts, indicates strongly a periodicity of the ovarian graft in the male. Unfortunately, however, it was almost impossible to form any judgment in regard to the regularity of the periodicity by the experimental method involved. A final solution of this question also must await further work. So far as they go the results point definitely to a periodic function on the part of the ovarian graft in males.

The problem of lactation will be considered more adequately in another paper. It is sufficient for the purposes here to re-emphasize the almost total absence of lactation in mammary glands in the male bearing ovarian grafts, despite the much greater activity than exists in the normal non-lactating female. The proper stimulus for lactation is not provided, but this cannot be dispensed with by assuming the cause to rest wholly with the absence of lutein tissue. It should be recalled that castrated males injected with lutein-free oestrin and subsequently with alkaline extracts of pituitary secrete copiously despite the fact that ovarian tissue had never been present in the animal. Again, this is not an argument against some influence from lutein tissue on lactation in the normal female, but it brings into question the absolute necessity of lutein secretions for lactation.

The final point for consideration and emphasis is the effect of the ovarian graft on the secretion of testis hormone in experimental cryptorchid males. That the oestrin secreted by the ovarian graft, or oestrin introduced by injection, does lessen the secretion of testis hormone in such animals is scarcely to be doubted if close attention

is given to the evidence presented. These observations on the effect of oestrin in the male guinea pig are entirely in line with the observations of Moore and Price on the rat. They are readily interpreted, as these authors have pointed out, on the basis of an inhibitory action of oestrin on the hypophysis, which reduces the available amount of the sex-stimulating substances for testis activity. The correctness or inadequacy of this explanation must await the collection of further evidence.

SUMMARY AND CONCLUSIONS

1. The mammary glands of male and female guinea pigs are somatic, non-sex-dimorphic, equipotential characters. They respond equally in the two sexes to the injection of oestrin.

2. There is no antagonism between oestrin and testis hormone. Oestrin alone, or injected along with effective doses of testis hormone, brings forth similar quantitative responses in the mammary glands of both sexes.

3. Ovary grafts in normal males persist but do not secrete oestrin. Abdominal elevation of the testes or castration permit the graft to secrete oestrin with mammary response greater than in normal or ovarian-grafted females.

4. The explanation of differences in mammary responses in males and females does not involve direct interaction of sex hormones or hormone antagonism. It involves the reciprocal gonad-hypophysial interactions.

5. Ovary grafts in experimental cryptorchid or castrated males appear to be periodic in function; this is indicated by the responses of endometrial grafts in the anterior chamber of the eye, and by oestrin excretion.

6. Lactation in males, though not usually occurring from influence of the graft alone, does occur when extracts of pituitary are administered; corpus luteum secretions are not essential for lactation.

7. The physiological capacity of the hypophysis differs in the two sexes. This difference appears to express a sex-limited character.

8. Ovarian grafts in experimental cryptorchid males reduce the intensity of testis hormone secretion by virtue of oestrin interference with hypophysis activity.

9. Sex hormone antagonism appears not to exist, at least in post-pubertal animals. The reciprocal gonad-hypophysial influences that exist in the normal organism for the control of the state of function of the gonads and accessory organs, and to some extent the anterior lobe of the hypophysis, offer a logical explanation for the activity of ovarian grafts and mammary gland responses.

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THE MORPHOLOGY AND PHYSIOLOGY OF THE SALAMANDER THYROID GLAND. VIII. FURTHER EXPERIMENTS ON THE THYREO-ACTIVATOR

(Two plates)

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IN SEVERAL articles (Schwartzbach and Uhlenhuth, 1928 and 1932) we reported experiments on small thyroidectomized larvae of the eastern and Utah races of the tiger salamander. Whereas it is easy to produce metamorphosis in the normal larvae of these salamanders by intraperitoneal injections of extracts of the beef prehypophysis, it is impossible to produce the same results after thyroidectomy. For this reason and, because the thyroids of the normal larvae are activated by the anterior-lobe injections in a specific manner, we concluded that the anterior lobe of the beef hypophysis contains a specific thyroid-activating hormone, the "thyreo-activator."

Although, contrary to Crew and Wiesner (1930), Paal (1931), and others, we consider these experiments as entirely conclusive, it appeared wise to repeat the anterior-lobe injections on thyroidectomized larvae at a stage which would be more comparable to the Mexican axolotl employed by Spaul (1930) in experiments which, contrary to our own experiments, seemed to demonstrate that anterior-lobe injections provoke metamorphosis even in the absence of the animal's own thyroid gland.

We are able to report in the present article on experiments performed on large larvae of the Colorado axolotl (body length of about 70–95 mm.), a race of *Ambystoma tigrinum*, which very closely resembles the Mexican axolotl. Moreover, in these new experiments we injected not only thyroidectomized larvae but, in addition, larvae from which the entire hypophysis and, in another lot, the hypophysis as well as the thyroid had been removed. Furthermore, to prove the effects of the thyreo-activator, the results of histological exami-

nations of the thyroids will be submitted. And, final'y, certain specific effects of the anterior lobe upon the weight of the animals will be discussed.

Anticipating the presentation of details, it may be mentioned here that again in no case could metamorphosis be evoked by injection of anterior lobe, after the thyroids had been extirpated, while all animals possessing their thyroids metamorphosed promptly when injected with the thyreo-activator, regardless of the presence or absence of the hypophysis.

MATERIAL

The Colorado axolotls used in these experiments were collected by Professor Gilmore[1] in the vicinity of Colorado Springs and were received in our laboratory October 17, 1931. On the same day the entire stock was placed into earthenware crocks filled with distilled water and kept in a Frigidaire at a temperature of 6–10° C. On November 23, 1931, the entire stock was transferred to glass jars; each animal was kept in an individual glass jar. From this time on the animals were kept at room temperature varying between 12 and 18° C.; instead of distilled water ordinary tap water was used. No food of any kind was offered during the entire period of observation.

When the animals arrived, they were in a completely larval stage, ranging in size from about 60 to 80 mm. body length. From laboratory stock reared at different temperatures as well as from observations in the natural habitat of the animals, it can be estimated that these larvae had hatched in the spring of 1930 and were approximately one and a half years old.

After the animals had been in the laboratory for several days, distinct signs of a beginning metamorphosis were noticed, provoked evidently by the change of the environment (disturbances due to the capture and transportation, increased temperature, cessation of feeding). Placing the animals in the icebox at low temperature, however, stopped the further progress of metamorphosis; as seen by the behavior of the controls (*a* 3 and *a* 4), metamorphosis progressed at a

[1] The authors wish to express their gratitude to Professor Gilmore of Colorado College, for his kindness in collecting the Colorado axolotls used in the experiments described in this article.

very slow rate; *a* 3 was still completely larval January 5, 1932, and *a* 4 began shedding skin February 3, 1932, i.e., after having been kept in the laboratory for $3\frac{1}{2}$ months.

TECHNIQUE OF OPERATION

The hypophysis was removed through the mouth, by the transphenoidal route. In chloretone anesthesia the animals were placed in a holder, which maintained them floating, on the back, in a 30 per cent frog Ringer solution, but at the same time kept the head in a fixed position and the mouth open. The rays of a Leitz arc lamp were directed, through a glass tube inserted into the gill slits of one side, upon the basisphenoid. The entire operation was carried out under a Zeiss dissecting microscope. After raising a flap of the dorsal wall of the mouth from the basisphenoid, a dental drill was used to cut out a hole corresponding in size and location to the infundibulum. With a pair of fine forceps and scissors the entire hypophysis (neural as well as glandular) was removed, care being taken that the blood supply to the brain, especially the basilar artery, was left intact.

After the operation the animals were placed into a weak solution of mercurochrom. In several instances the entire brain started to undergo a slow disintegration believed to be due to a disturbance of the blood supply. These animals succumbed ultimately and will not be considered in this article.

The thyroids were removed under 30 per cent frog Ringer solution and in chloretone anesthesia. A median incision was made through the skin and floor of the opercular pouch extending from the pectoral region to the tip of the lower jaw. The skin was pulled laterally on both sides and the field of operation exposed widely. The geniohyoid muscles were raised from the sternohyoid muscles; this procedure made it possible to pull the geniohyoid muscles freely either laterally or medially and to examine thoroughly, with the aid of a Zeiss binocular dissecting microscope (magnification 13 and 33), the intermuscular spaces. The blood vessels, especially the external jugular veins, were carefully searched along their entire course. After the thyroids were located, they were removed, under the microscope, together with a considerable portion of the blood vessels to which

they were found attached. In this particular stock not more than one lateral thyroid on either side was found; median thyroids were present only in a few animals.

PREPARATION OF POWDERS

The anterior-lobe powders were prepared in the way described in previous articles. Altogether, three different powders were used, each one made from ox glands.[2] Special care was taken that each powder was used not only in experiments in which the powders, owing to the nature of the experiment, were ineffective but also in experiments in which they should have been—and were—effective; in this manner the effectiveness of each powder was tested out, and we could feel assured that, in those experiments in which no response was obtained, the ineffectiveness of the powders was not due to the nature of the powder but to the special procedure of the experiment.

In two control animals *a* 3 and *a* 4, powder of calf and beef muscle were employed. Both were prepared exactly like the anterior-lobe powders.

PREPARATION OF EXTRACTS AND DOSAGE

The powders were extracted in the manner described in previous articles. As an extractive, 0.5 per cent glacial acetic acid in 30 per cent frog Ringer was used. In this series of experiments special care was taken that the suspension of the powder was heated immediately after the powder had been added to the extractive. Immediately after the suspension had been brought to a boil, it was poured on the filter. This latter procedure was adopted, as in previous experimentation it was suspected that, if the suspension is allowed to stand after boiling, a quantity of the hormone is adsorbed to the particles and does not pass the filter. The filtrate of the anterior-lobe extract is entirely clear and of a pink color. Immediately after filtration the fluid is neutralized with N/5 NaOH, against litmus paper.

The quantity of powder used was such as to give, after neutralization, an extract of 100 mg. substance per 0.5 cc. of fluid.

[2] The authors take pleasure in expressing their appreciation to Mr. Robert Fox, of the Greenwald Meat Packing Company of Baltimore for permission to work in the abattoirs of the company and for most generous assistance received during our work from the employees of the Company.

The injections were made intraperitoneally, three times a week; 0.5 cc. of fluid was used per injection and animal. The injections were made in chloretone anesthesia.

Inspection of Table I shows that in no case more than 6 weeks were allowed to elapse between the operation and the first injection.

EXPERIMENTS

The entire experiment (CCCXXVII) consisted of 5 series; normal control animals (*a*), normal animals injected with anterior lobe (*b*), thyroidectomized animals injected with anterior lobe (*c*), hypophysectomized animals injected with anterior lobe (*d*), and thyreohypophysectomized animals injected with anterior lobe (*e*). The sizes (body length) of the individual animals, at the beginning of the experiment, are recorded in Table II.

I. EFFECT OF ANTERIOR-LOBE INJECTIONS UPON METAMORPHOSIS

a) *Normal controls.*—Animals *a* 1 and *a* 2 were killed for histological examination of the endocrine organs, at the beginning of the experiment. Both animals are representative of the entire stock used for experimentation. They were completely larval, measuring about 80 mm. body length.

Animals *a* 3 and *a* 4 received control injections; each animal received 2 Ringer injections (0.5 per cent glacial acetic acid in 30 per cent frog Ringer, neutralized with N/5 NaOH against litmus paper) and 7 calf-muscle injections; *a* 3, in addition, 6 beef-muscle injections, *a* 4, 24 beef-muscle injections.

Animal *a* 3 was completely larval when killed (after 15 injections); *a* 4 shed his skin for the first time after the twenty-sixth injection; when killed, after 33 injections, he was far advanced in metamorphosis, his gill slits closed, and the gills reduced to small stubs. Although he metamorphosed spontaneously, metamorphosis did not occur until $3\frac{1}{2}$ months after the animals had been received in the laboratory.

b) *Normal anterior-lobe-injected animals.*—Both animals, *b* 1 and *b* 2, received injections of anterior-lobe extract (specimen E.U., 18.IX.31, No. 2). Both animals started shedding skin after the sixth injection. Animal *b* 1 was killed after the eleventh injection, *b* 2 after the twelfth injection. In both animals the gill slits were

TABLE I

SUMMARY OF ENTIRE EXPERIMENT

Serial Number	Date of Operation	Date of First Injection	Total Number of Injections	Duration of Experimental Period in Days	Number of Powder from Which the Extract Was Made*	Date at Which the Experiment was Terminated	Number of Anterior-Lobe Injections Required To Produce Skin Shedding	Number of Anterior-Lobe Injections Given without Producing Any Metamorphic Effect	Developmental Stage at Time of Death
Controls, Muscle Injected									
a 3	.....	Dec. 5, '31	15	31	Muscle	Jan. 5, '32	.....	.....	Completely larval
a 4	.....	Dec. 5, '31	33	73	Muscle	Feb. 16, '32	.....	.....	Shed skin, slits closed. Gill stubs
Controls, Anterior Lobe Injected									
b 1	.....	Nov. 23, '31	11	19	1	Dec. 12, '31	6	.....	Shed skin, slits open, gill stubs
b 2	.....	Dec. 5, '31	12	24	1	Dec. 29, '31	6	.....	Shed skin, slits open, gill stubs
Thyroidectomized, Anterior Lobe Injected									
c 2	Nov. 17, '31	Dec. 7, '31	32	71	1, 2, 3	Feb. 16, '32	.....	32	Completely larval
c 3	Nov. 17, '31	Dec. 7, '31	34	75	1, 2, 3	Feb. 20, '32	.....	34	Completely larval
Hypophysectomized, Anterior Lobe Injected									
d 1	Nov. 5, '31	Nov. 23, '31	12	21	1	Dec. 14, '31	11	.....	Shed skin
d 2	Nov. 17, '31	Dec. 7, '31	7	15	1	Dec. 22, '31	6	.....	Shed skin
d 7	Dec. 5, '31	Dec. 14, '31	15	33	1, 2	Jan. 16, '32	11	.....	Shed skin, slits partly closed, gill-nodules
d 8	Dec. 5, '31	Dec. 21, '31	12	26	1, 2	Jan. 16, '32	8	.....	Shed skin, slits partly closed, gill-nodules
d 9	Dec. 5, '31	Jan. 18, '31	15	33	1, 2, 3	Feb. 20, '32	7	.....	Shed skin, slits partly closed, gill-stubs
Hypophyseo-thyroidectomized, Anterior Lobe Injected									
e 1	Oct. 23, '31 Nov. 20, '31	Dec. 7, '31	37	82	1, 2, 3	Feb. 27, '32	.....	37	Completely larval
e 6	Nov. 5, '31 Nov. 20, '31	Dec. 7, '31	34	75	1, 2, 3	Feb. 20, '32	.....	34	Completely larval

* Anterior-lobe powder No. 1 (E.U. 18.IX.31, No. 2) was used until January 4, 1932; powder No. 2 (E.U. 18.IX.32) was used from January 6, to January 25, 1932; powder No. 3 (U. and F., 8.I.32) was used from January 27, 1932, to the end of the experiment.

still open, but the gills were reduced to short stubs. In *b* 1 the gill filaments had completely disappeared.

To produce skin shedding, 6 injections of anterior lobe were required. After 11 injections metamorphosis was in full progress. In *b* 1 it started 2 months, in *b* 2, 1½ months, before it started in the control animal *a* 4 and was therefore distinctly accelerated by the anterior-lobe injections.

TABLE II

SIZE OF INDIVIDUAL ANIMALS AT THE BEGINNING OF THE EXPERIMENT

Serial Number of Animal	Body Length* in Mm.
a 1	86.0
a 2	72.7
a 3	75.0
a 4	82.0
b 1	77.0
b 2	87.0
c 1	....
c 2	92.0
c 3	89.0
c 4	96.0
d 1	90.0
d 2	90.0
d 7	83.0
d 8	83.0
d 9	87.0
e 1	87.0
e 6	92.0
e 8	86.0
e 9	81.0
e 10	83.0

* From tip of upper jaw to posterior commissure of external cloacal orifice.

It must be kept in mind, of course, that the anterior-lobe injections were not started immediately after the animals had been received in the laboratory; otherwise the acceleration of the metamorphosis of the anterior-lobe-injected larvae, as compared to the controls, would have been even greater.

c) *Thyroidectomized animals.*—Four animals, *c* 1, *c* 2, *c* 3, *c* 4, entirely comparable to the anterior-lobe-injected larvae *b* 1 and *b* 2, were thyroidectomized. Of these, *c* 4 was kept as a control animal and at the time of writing this article (nearly 1 year after the beginning of the experiment) it is still fully larval. Animals *c* 2 and *c* 3 both received injections of anterior lobe. Although *c* 2 received 32 and *c* 3 received 34 injections, no signs of metamorphosis could be produced; when the animals were killed, after the last injection, they resembled much the uninjected thyroidectomized control *c* 4, except that the injected animals were deep black.

It is noteworthy that at the time when these animals were still fully larval, even the control animal *a* 4 had metamorphosed.

For the first 14 injections the same specimen of anterior lobe had been used, which metamorphosed the animals *b* 1 and *b* 2; for the remaining injections two specimens of anterior lobe (E.U., 18.IX.31, No. 1, and U. and F., 8.I.32) were used which proved potent in the case of the next experiment (*d* 7, *d* 8, and *d* 9).

Careful examination of the hyoid region revealed the presence of a small thyroid remnant in *c* 3; it consisted of only four small follicles and was discovered only by its conspicuous blood supply. In *c* 2 no thyroid remnant could be found.

Thirty-four injections of potent anterior-lobe extract did not produce any metamorphic changes in the thyroidectomized larvae.

d) *Hypophysectomized larvae.*—Of 9 hypophysectomized larvae, *d* 1, *d* 2, *d* 7, *d* 8, and *d* 9 survived, all of which received injections of anterior-lobe extract. Animal *d* 1 started shedding skin after the eleventh injection; he died after the twelfth injection at which time only initial metamorphic changes had taken place. Animal *d* 2 started skin shedding after the sixth injection; he died after the seventh injection, at which time only initial metamorphic changes had taken place. Animal *d* 7 started skin shedding after the eleventh injection; he was killed after the fifteenth injection; at that time metamorphosis was far advanced, the gill slits were partly closed, and the gills were reduced to small black nodules. Animal *d* 8 started skin shedding after the eighth injection; he was killed after the twelfth injection, in a state of advanced metamorphosis; the gill slits were partly closed, the gills

were reduced to small black nodules. Animal *d* 9 started shedding skin after the seventh injection; he was killed after the fifteenth injection, in a state of advanced metamorphosis; the gill slits were partly closed, the gills reduced to short stubs without fringes. Histological examination of the brain proved the complete absence of the hypophysis.

Again, it is noteworthy that all of the hypophysectomized larvae started metamorphosing before the control animal *a* 4. This is especially significant, as the injections of anterior lobe had been started quite late. In *d* 7 and *d* 8 anterior-lobe injections were started after the first 2 animals of the series, *d* 1 and *d* 2, had been forced into metamorphosis by the injections. And, in *d* 9, injections were not started until *d* 7 and *d* 8 had been metamorphosed, i.e., 3 months after the animals had been received in the laboratory. Briefly, then, three successive sets of hypophysectomized larvae were metamorphosed by the anterior-lobe injections during a period of time which was insufficient to allow the control animal *a* 4 to metamorphose. In particular, the animal *d* 9 was not injected until the end of the third month after arrival in the laboratory and not until the end of $1\frac{1}{2}$ months after operation. During this entire time he remained completely larval; the more conspicuous was his transformation after injections had been started.

Six to 11 injections of anterior lobe are sufficient to cause skin shedding in hypophysectomized animals; 12–15 injections produce a state of advanced metamorphosis. The hypophysectomized animals react to anterior-lobe injections much like normal animals.

e) *Thyreo-hypophysectomized larvae.*—Of the 9 larvae deprived of both the thyroid and hypophysis, *e* 1 and *e* 6 received anterior-lobe injections. Animal *e* 1 was killed after 37, *e* 6 after 34, injections; at that time neither animal showed any signs of metamorphosis. Careful examination of the hyoid region failed to reveal any trace of thyroid tissue; histological sections of the brain proved that the hypophysis had been removed completely. For the anterior-lobe injections the same specimens of anterior-lobe powder had been used which proved potent in all those animals which were in the possession of a normal thyroid gland. Moreover, in the case of *e* 1 the dose of anterior lobe was increased when it was seen that the animals

did not respond to the injections. At the last 3 injections (35th, 36th, and 37th) 160 mg. instead of 100 mg. of anterior-lobe powder were injected.

It is evident that animals, from which both thyroid and hypophysis are removed, cannot be metamorphosed by injections of anterior lobe; in this respect these anima'ls behave like larvae which are deprived of the thyroid gland only.

Summarizing the effects of anterior-lobe injections upon the metamorphosis of the Colorado axolotl, it may be stated that metamorphosis cannot be produced in the absence of the thyroid gland, while, in all animals possessing a thyroid, metamorphosis is promptly evoked by the injections of anterior lobe, no matter whether or not the hypophysis is present.

THE EFFECTS OF ANTERIOR-LOBE INJECTIONS UPON THE BODY-WEIGHT OF THE ANIMALS

It is well known that the larvae of amphibians show a considerable decrease in length as well as in weight during metamorphosis. This loss of weight is generally attributed to the activity of the thyroid gland in metamorphosis and is considered homologous to the wasting which takes place in hyperthyroidism of mammals.

It would be expected that a decrease in weight comparable to that which occurs in normal metamorphosis and in metamorphosis caused by experimental administration of thyroid hormone should take place in the larvae which are metamorphosed by the injection of anterior-lobe substance. Such is actually the case, as may be seen from an inspection of Table III.

For the same reason we expected that thyroidectomized larvae would remain constant in weight when injected with anterior lobe. Contrary to this expectation, however, both the thyroidectomized as well as the thyreo-hypophysectomized animals (*c* and *e*) show very much the same loss of weight when injected with anterior lobe as do the larvae possessing a thyroid gland.

That this decrease in weight was not caused by starvation (no food was administered to the animals) is evident from the fact that the thyreo-hypophysectomized larvae *e* 8, *e* 9, *e* 10, which did not receive anterior-lobe injections and were kept at the same tempera-

ture as the injected animals, showed none or only an insignificant loss of weight during the same period of time, during which the injected animals of this group decreased more than 30 per cent in weight. After the termination of the experiment one of the unin-

TABLE III

THE LOSS OF WEIGHT IN THE THYROIDECTOMIZED LARVAE CAUSED BY ANTERIOR-LOBE INJECTIONS

(Body Weight in Gm.*)

	At the Beginning of the Experiment, Before Operation and Injections	1½ Mos. after Operation	At the End of Experiment (after Injections)	Nearly 1 year after Arrival in Laboratory Sept. 14, 1932	Difference in Weight between Beginning and End of Experiment, in Percentages of Initial Weight
a 1....	20.1	.....	.....	.....	.....
a 2....	15.0	.....	.....	.....	.....
a 3....	(larval) 18.0	.....	17.2	.....	4.4
a 4....	20.4	.....	13.5	.....	33.8
b 1....	19.4	.....	12.0	.....	38.1
b 2....	26.0	.....	19.7	.....	24.3
c 2....	33.8	.....	23.5	.....	30.5
c 3....	29.5	.....	20.0	.....	32.2
d 1....	(not fully metamorphosed) 35.0	.....	34.2	.....	2.3
d 2....	(not fully metamorphosed) 25.8	.....	29.6	.....	.....
d 7....	19.5	.....	12.5	.....	35.9
d 8....	21.4	.....	14.2	.....	33.6
d 9....	26.0	29.2	17.0	.....	41.8
e 1....	26.2	.....	18.0	.....	31.3
e 6....	34.2	.....	22.5	.....	34.2
e 8....	(uninjected) 25.2	.....	25.5	24.2	0.7
e 9....	(uninjected) 20.0	.....	19.0	.....	5.0
e 10...	(uninjected) 22.2	.....	21.0	.....	5.4

* Weights are taken by placing the animals in a weighed dish of water.

jected animals of group *e* was placed into the refrigerator (at 6–10° C); when weighed recently (nearly 1 year after the animal arrived in the laboratory) it had lost only a little more than 1 gm. of its original weight.

The loss of weight after anterior-lobe injections is particularly conspicuous in those larvae which are hypophysectomized before injections are started. For in those animals, a distinct and significant

increase in size, easily noticeable without special measurements, takes place soon after the hypophysectomy.

One of the animals of group *d* (*d* 9) was weighed 1½ months after hypophysectomy and before the injections of anterior lobe had been started; inspection of Table III shows that he had increased in weight by more than 3 gm. (i.e., about 11 per cent).

We are unable to explain, at present, these changes of the weight of the animals, and, more particularly so, the loss of weight of the thyroidectomized larvae, caused by anterior-lobe injections. As we had no opportunity to test our theories concerning these observations, we have to confine ourselves to merely recording them here as facts.

THE EFFECT OF THE THYREO-ACTIVATOR UPON THE STRUCTURE OF THE THYROID GLAND

In 6 normal larval animals in which the thyroids were removed immediately after the animals had been taken out of the refrigerator, the thyroids showed no distinct signs of activity. The follicles are of uniform size, of regular ovoid shape, and closely applied to each other; the interfollicular spaces are narrow and inconspicuous. The epithelium is low cuboid or flat. Intracellular colloid droplets and Andersson vacuoles are absent or insignificant in number. Colloid vacuoles are completely absent.

In one control larva (*a* 3) in which the thyroids were removed after 15 control-injections (Ringer solution and muscle extract) and more than 1 month after transfer from the refrigerator into room temperature, the thyroid shows but little activity. The size of the follicles is more variable than in the previous group, but the shape of the follicles is again mostly regular ovoid, although some folding is found. The interfollicular spaces again are extremely narrow. The epithelium is varying in height from flat to cuboid. The cytoplasma contains ordinary cell granules of great definition, but in small numbers and of very small size; in Champy preparations stained with Heidenhain-Mallory these stain bright red. Colloid droplets and Andersson vacuoles are completely absent. The intrafollicular colloid contains small vacuoles in small numbers.

The control animal *a* 4, which metamorphosed spontaneously 3½ months after arrival in the laboratory and 2½ months after being transferred from the refrigerator into the ice box, is entirely compa-

rable to the animals metamorphosed by injections of thyreo-activator, as far as its physiological state is concerned. The follicular pattern of this animal is only slightly different from that of the preceding animals. The follicles are of rather uniform size and mostly of regular ovoid shape; only few follicles are collapsed. The interfollicular spaces are narrow. The cells are higher and larger than in the larval control glands, mostly high cuboid in shape. The cytoplasma is charged with numerous small granules of spherical shape, staining purplish or red in the Champy preparations. In addition, slightly larger granules are found which stain dark blue; the latter are possibly small colloid droplets. Larger colloid droplets are completely absent. The vacuolization of the cells is only slight and is quite characteristic in as much as it concerns only the basal portion of the cell and is frequently represented by a large fluid area in which the nucleus seems suspended. The colloid does not contain any vacuoles.

In both the normal animals which had received injections of thyreo-activator (*b* 1 and *b* 2), the thyroids are considerably activated as compared to the uninjected controls and show the same characteristics in both animals. The follicles are mostly completely collapsed, containing only a slit-like lumen. In each section only a few individual small follicles are present, while the rest of the epithelium is represented by a single mass of epithelium forming a continuous network. The intrafollicular spaces are very large and filled with quantities of blood. The epithelium is very high, mostly columnar. In addition to large numbers of ordinary cell granules the cytoplasma contains in many cells one or several intracellular colloid droplets. These are located in the apical parts of the cells and show frequently a peculiar association with a vacuole indicating that the droplet is in a state of being dissolved. The basal cell ends exhibit a high degree of vacuolization; the liquid is contained not in spherical, well-defined vacuoles, but in large irregular lacunae. It is noteworthy that the intrafollicular colloid contains no vacuoles.

In the 3 hypophysectomized animals which received injections of thyreo-activator (*d* 7, *d* 8, and *d* 9), the thyroids are again activated. While they show the same characteristics in all 3 animals, they are different from the activated thyroids of the non-hypophysectomized animals *b* 1 and *b* 2. The follicles, quite variable in size, are not collapsed, but of a regular, ovoid shape, and do not form a continuous mass of

epithelium. The interfollicular spaces are very narrow. The intrafollicular colloid contains large numbers of large vacuoles, a circumstance which makes these thyroids immediately conspicuous among all the other thyroids. The epithelium is high, often columnar, but generally not as high as in the glands of the previous group. The number of ordinary cell granules is very high; among them are always conspicuous a few darker granules, of great definition and easy to count. In Champy preparations these stain red. Intracellular colloid droplets are rare. Andersson vacuoles are found in small numbers and occupying the apical portions of the cells.

On Plate I the outlines of representative Zenker sections of a normal larval control animal (*a* 3), of a normal metamorphosed control animal (*a* 4), of a normal anterior-lobe-injected animal (*b* 1), and of a hypophysectomized anterior-lobe-injected animal (*d* 7) are illustrated, to show the follicular pattern. On Plate II representative cell types (Zenker) of the same thyroids are illustrated.

Summarizing the effect of the thyreo-activator upon the structure of the thyroid the following statements seem pertinent. The thyroids of all the animals metamorphosed by injection of thyreo-activator are more active than the thyroids of normal larval animals and of larvae which metamorphosed spontaneously. The structure of the thyroids of hypophysectomized animals injected with thyreo-activator, however, is remarkably different in some respects from the thyroid structure of normal animals injected with thyreo-activator. We do not as yet understand the significance of this difference, but it appears that in the latter group the cells are predominantly absorbing the intrafollicular colloid through the apical ends in the form of stainable colloid droplets and excreting a non-stainable fluid at the base, causing thus an emptying of the follicles, while in the hypophysectomized animals the cells are predominantly excreting an unstainable fluid into the follicular lumen, causing thus a distension of the follicles.

But whatever the difference may mean, it is certain that it is caused by a difference in the physiological state of the animals and not by a difference in the procedure of injecting. Both groups of animals were kept under exactly the same conditions. The same specimens of powders and extracts were used and, at least in some animals, the number of injections were the same.

PLATE I

Outline drawings of representative sections of normal and activated thyroid glands. Drawn with camera lucida, Ocular Zeiss ×10, Objective Zeiss ×10.

The gray area indicates the epithelium of the wall of the follicles, the white area inclosed by the gray circles indicate the lumen of the follicles, the white area marked with "*b*" indicates blood spaces between the follicles, the black circles indicate location, size, and number of colloid vacuoles. Thyroids fixed in Zenker-glacial.

a 3, thyroid of a normal larval control animal.

a 4, thyroid of a normal metamorphosed control animal.

b 1, thyroid of a normal animal metamorphosed by injections of thyreo-activator.

d 7, thyroid of a hypophysectomized animal metamorphosed by injections of thyreo-activator.

PLATE II

Camera lucida drawings of representative cells of normal and activated thyroid glands of same glands illustrated on Plate I.

All cells are drawn at the same scale (ocular, Zeiss ×10; Objective Spencer 1.8 mm.). The thyroids were fixed in Zenker-glacial.

a 3, cuboidal cells showing no signs of activity.

a 4, high cuboidal cells showing an apical zone of dense cytoplasma and basal vacuolized zone.

b 1, *A*, high cylindrical cells containing stainable colloid droplets at the lumen side and unstainable lacunae at the base; *B*, high cylindrical cells containing dissolving colloid droplets at the lumen side and lacunae at the base.

d 7, *A*, high columnar cells; *B*, columnar cells containing a stainable colloid droplet at the lumen side; *C*, high cuboidal cells containing unstainable vacuoles at the lumen side; *D*, columnar cells excreting vacuoles into the lumen of the follicle.

PLATE I

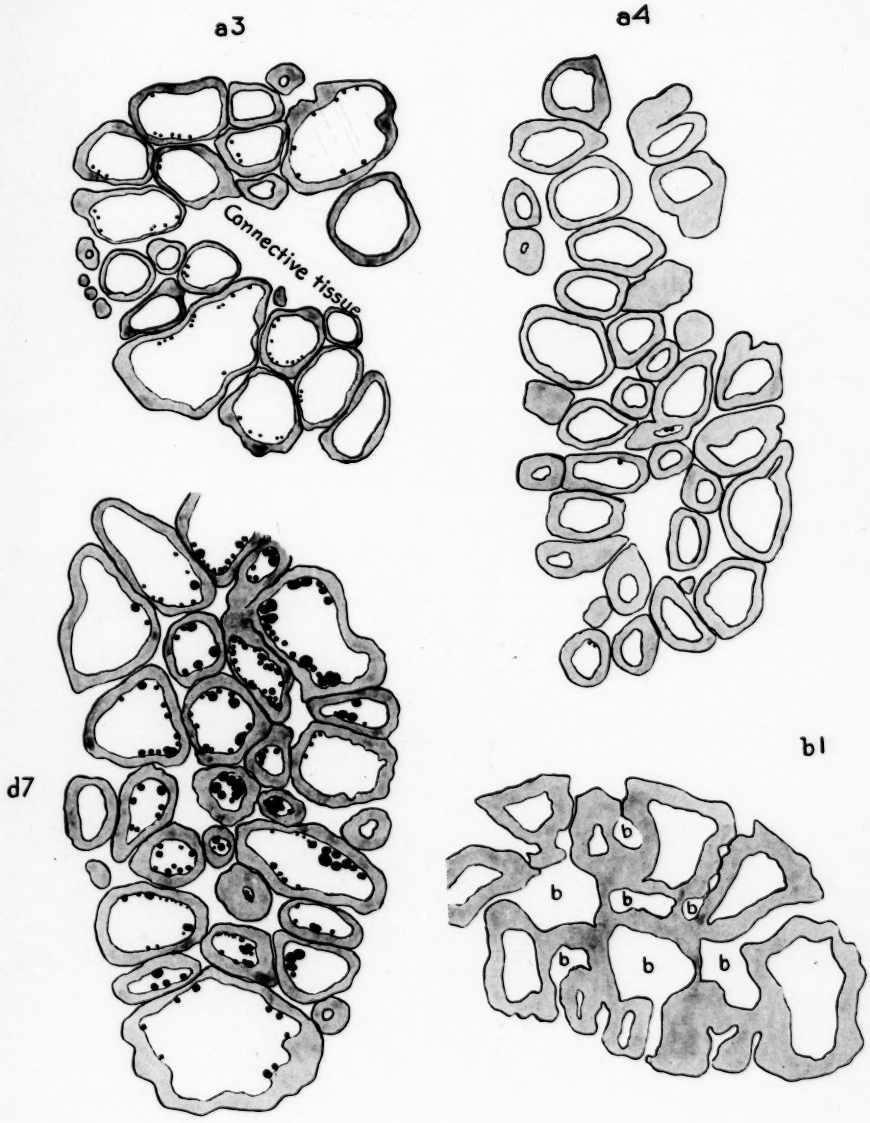
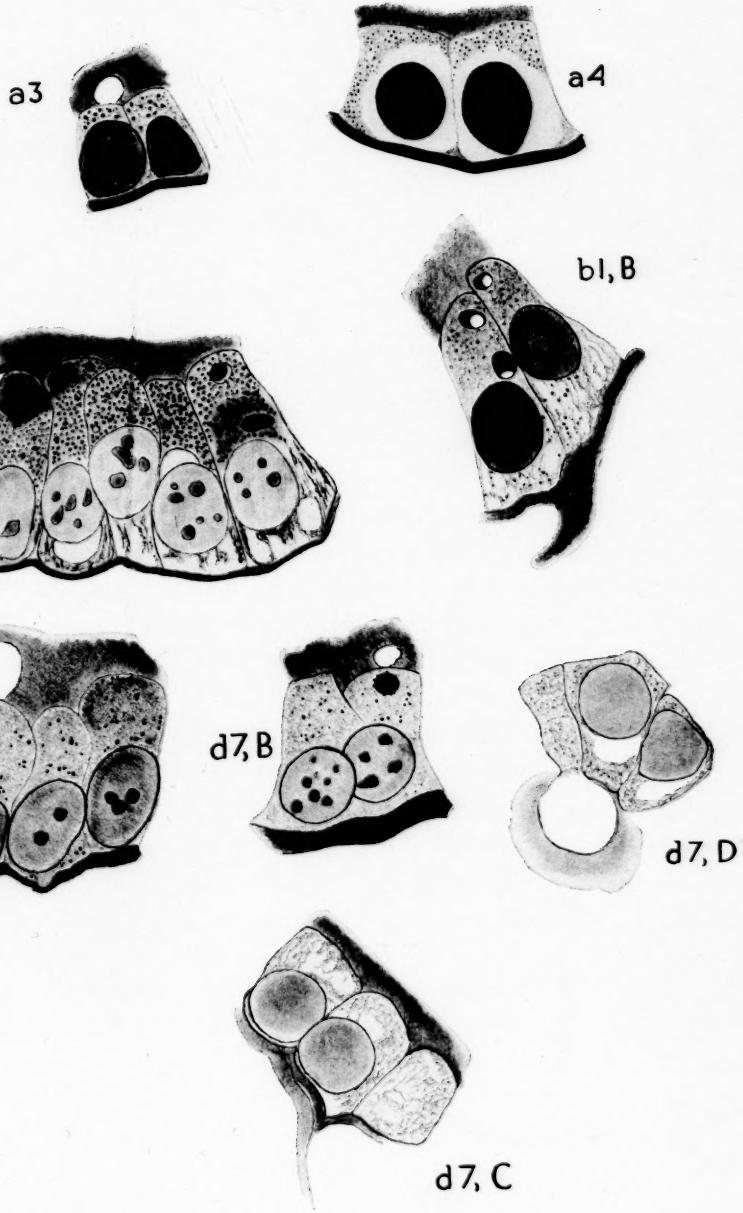


PLATE II



SUMMARY

1. Large Colorado axolotls, normal, thyroidectomized, hypophysectomized and thyreo-hypophysectomized are injected with thyreo-activator extracted from the anterior lobe of beef hypophysis, and their response is compared to the behavior of normal axolotls injected with calf- and beef-muscle extract.

2. In all larvae possessing thyroid glands, regardless of the presence or absence of the animals' own hypophysis, metamorphosis is greatly accelerated by the injections of thyreo-activator, while it is impossible to metamorphose thyroidectomized larvae by the same kind of injections.

3. Removal of the hypophysis causes an increase in the body weight of the animals, while injections of thyreo-activator provoke a loss of weight in all animals, even in those from which the thyroids had been removed.

4. In all the animals possessing a thyroid the injections of thyreo-activator caused an activation of the thyroid cells. The structural picture of the thyroid resulting from it was remarkably different, however, in the two groups. The differences are described and illustrated in this article. While a final interpretation of this difference had to be postponed, it seems probable that in the normal anterior-lobe-injected animals the cells are predominantly engaged in emptying the follicles, while in the hypophysectomized anterior-lobe-injected animals the cells are predominantly active in the direction of filling the follicles.

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